CHEMICAL PROFILING AND BIOLOGICAL ACTIVITY ON LEAVES OF *OCIMUM GRATISSIMUM* GROWN IN TAMILNADU

Dissertation Submitted to

The Tamil Nadu Dr. M.G.R. Medical University,

Chennai – 600 032.

In partial fulfillment for the award of Degree of

MASTER OF PHARMACY

(Pharmaceutical Chemistry)

Submitted by

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Under the Guidance of

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(Accredited By "NAAC" with a CGPA of 2.74 on a Four Point Scale at "B" Grade)

Melmaruvathur-603 319

May - 2012

CERTIFICATE

This is to certify that the research work entitled **"CHEMICAL PROFILING AND BIOLOGICAL ACTIVITY ON LEAVES OF *OCIMUM GRATISSIMUM* GROWN IN TAMILNADU"** submitted to The Tamil Nadu Dr. M.G.R Medical University in partial fulfillment for the award of the Degree of Master of Pharmacy (Pharmaceutical Chemistry) was carried out by **A.PANDIYAN (Reg.No.26091873)** in the Department of Pharmaceutical Chemistry, Adhiparasakthi College of Pharmacy, Melmaruvathur, under my direct guidance and supervision during the academic year 2011-2012.

Place: Melmaruvathur

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A.PANDIYAN

CONTENTS

LIST OF ABBREVIATIONS

%	Percentage
^{o} C	Degree centigrade
g	Gram
mg	Milli gram
µg	Microgram
h	Hours
l	Litre
Min	Minutes
mm	Milli meter
nm	Nanometer
MIC	Minimum Inhibitory Concentration
ml	Milliliter
IR	Infrared Spectroscopy
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
GC-MS	Gas Chromatography-Mass Spectroscopy
GC-FID	Gas Chromatography-Flame Ionization Detector
TLC	Thin Layer Chromatography
LD	Lethal Dose
DPPH	1,1-DiPhenyl-2-PicrylHydrazyl
RAPD	Random Amplified Polymeric DNA
SPME	Solid Phase Micro Extraction
PCR	Polymerase Chain Reaction

Ha	hectare
ELISA	Enzyme-Linked Immuno Sorbent Assay
HCl	Hydro chloric acid
H_2SO_4	Sulphuric acid
Conc.	Concentrated
$CHCl_3$	Chloroform
KI	Potassium Iodide
CFU	Colony Forming Unit
HNO_3	Nitric acid
NH_3	Ammonia
$HgCl_2$	Mercuric chloride
NaOH	Sodium hydroxide
CCl_4	Carbon tetra chloride.

1. INTRODUCTION

Natural plant products and their analogues are an important source of drugs for centuries and also they are used in traditional treatments to cure variety of diseases. In the last few decades there has been an exponential growth in the field of herbal medicine. In many parts of the world medicinal plants are used against bacterial, viral and fungal infections. Practitioners of Ayurveda and Unani system of medicine regularly employ a large number of Indian medicinal plants as anti-biotic agents and over the last 40 years, intensive efforts have been made to discover clinically used herbal anti-bacterial and anti-fungal drugs. Medicinal plants have been used by human and their components are widely used in medicine as being since ages in traditional medicine due to their constituents of different medical products, in the food therapeutic potential and the search on medicinal plants industry as flavoring additives and also in cosmetics as have led the discovery of novel drug candidates used fragrances.

As per WHO, Traditional medicines are the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses.

Herbal medicines include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant materials as active ingredients, having wide spectrum of biological activities.

Phytochemical is a natural bioactive compound found in plants, such as vegetables, fruits, medicinal plants, flowers, leaves and roots that work with nutrients and fibers to act as an defense system against disease or more accurately, to protect against disease. Phytochemicals are divided into two groups, which are primary and

secondary constituents; according to their functions in plant metabolism. Primary constituents comprise common sugars, amino acids, proteins and chlorophyll while secondary constituents consists of alkaloids, terpenoids and phenolic compounds and many more such as flavonoids, tannins and so on.

The therapeutic effect of the drugs are due to the presence of low volume and high value of secondary matabolites such as alkaloids, terpenoids, saponins, steroids, coumarins, tannins, flavonoids and other phenolic compounds.

The potential of the phytochemicals have large scale pharmacological and biological activities such as anti-oxidant constituents (hydrolysable tannins, phenolic acid and flavonoids) of the plant materials for the care of health and protection from coronary heart diseases, cancer, anti-carcinogenic and anti-mutagenic effects. Varieties of herbaceous vegetables are protective against various diseases, particularly cardiovascular diseases. These herbaceous plants and species are harmless sources for obtaining natural anti-oxidants. Anti-oxidant constituents can delay or inhibit the oxidation of lipids and other compounds by inhibiting the propagation of oxidation chain reaction. Primarily, anti-oxidant effect is due to phenolic compounds such as phenolic acid, flavonoids and phenolic diterpenes and their mode of action for anti-oxidant compounds is due to its redox reaction properties which can absorb and neutralize free radicals by quenching singlet and triplet oxygen. Human diet does not always contain the required amount of vitamins for the normal growth and maintenance of the body function and as such cannot produce enough quantity for their body metabolism, so it can be obtained from fruits, vegetables and foods. Deficiency of vitamins can cause serious human health diseases and sometimes, very small concentrations are required for maintenance of good human health.

In general, it has been observed that a single volatile oil invariably comprise even more than 200 different chemical components, and mostly the trace constituents are solely responsible for attributing its characteristic flavor and odor.

In fact, most of the new chemicals entities have been introduced by semi-synthetic modification of natural plants and further research in medicinal plants still continues a feasible source of discovery of novel drugs.

LITERATURE RIVIEW

2. LITERATURE RIVIEW

OCIMUM GRATISSIMUM Linn.,

2.1. History:

Generally the *Ocimum* is an aromatic medicinal herb, referred as Tulasi or basil, widely distributed in India and the word basil comes from greak with meaning of "King". There are more than 150 different varieties available in the *Ocimum* genus and which have the large number of species, subspecies extensively having the much of varieties in the form of botanical nomenclature.

Tulasi is the most sacred, culinary herbal plant and it is used by many Ayurvedic practitioners for many health ailments and having significance in spiritual. It is acclaimed as possessing sattva (energy of purity) and as being capable of bringing of goodness, virtue and joy in humans.

Ocimum gratissimum is one of the variety of *Ocimum* genus and it is known as Ram Tulasi, also called as clove basil, tree basil, shrubby basil, fever leaf, african basil. It is coming under the family of *Labiatae* (*Lamiaceae*), and the subfamily of *nepetoideae.*

The *O.gratissimum* is an important group of aromatic and medicinal plant which yields many essential oil, aroma chemicals and finds diverse uses in the perfumery and cosmetics industries as well as in indigenous systems of medicine. *Ocimum* species with oil rich in camphor, citral, geraniol, linalool, methyl chavicol, eugenol and thymol are important and can be harnessed for successfully utilization by the industries.

The Ram tulasi have been used as folk medicine against fever, inflammations of the throat, ears or eyes, stomach pain, diarrhea and skin diseases and it is being used as anti-biotic.

Fig. 1: Plant *Ocimum gratissimum*

Fig. 2: Leaf of *Ocimum gratissimum*

Vernacular Name (In India):

Tamil	:	Elumicha Tulasi
Malayalam	:	Kattuthrithava
Kannada	:	Elumicha Tulasi
Hindi	:	Ram Tulasi
Bengali	:	Ram Tulasi, Ban Tulasi
Sanskrit	:	Vridha Tulasi, Ajaka
Punjabi	:	Banjere
Marathi	:	Rama Tulasi
Telugu	:	Nimma Tulasi

Vernacular Name (In other countries):

English	:	Shrubby basil, Large basil
Nigeria	:	Alfavaca
Spanish	:	Albahaca de limon
Thai	:	Horapha-chang
Indonesian	:	Ruku-ruku rimba
Malay	:	Ruku-ruku hitam
French	:	Methane gobanoise

2.2. Origin and Distribution:

The plant *Ocimum gratissimum* found and distributed all over peninsular India, Bangladesh, African countries, Thailand, Haiti, Egypt and Indonesia. In India, it is found in Tamilnadu, Bengal, Orissa, Karnataka, Kerala, Jammu and Kashmir and the different species of *Ocimum* are well distributed in all over tropical area of India.

2.3. Description:

Ocimum gratissimum is an aromatic, perennial herb, 1-3 meter tall, stem erect, round-quadrangular, much branched, glabrous or pubescent, woody at the base, often with epidermis peeling in strips.

Leaves are always opposite, petioles 2-4.5 cm long, slender, pubescent, blade elliptical to ovate, membranaceous, sometimes grandular punctate, base cuneate. Flowers in 6-10 flowered verticillasters, small, hermaphrodite, calyx 2 lipped, 2-3 mm long.

Fruits are pubescent upper lip rounded and recurved, reflexed in fruit, lower lip with 4, narrow, pointed teeth, central pair of teeth minute and much shorter than the upper lip. It consisting one seeded nutlets enclosed in the persistent calyx nutlet subglobose, 1.5 mm long, rugose and brown in color. (Kritikar R.K *et al.*, 2005)

2.4. Propagation and Cultivation:

The *O.gratissimum* can be cropped up well under tropical climate upto an altitude of 1800 meter. The growth is poor in areas which receive heavy and continuous rainfall. Frost is harmful to the plant and hence frost prone areas are to be avoided. Basil can be cultivated on a wide variety of soils, though moderately fertile well drained loamy or sandy loam soils are considered ideal for its cultivation. Basil is tolerant to higher concentration of copper and zinc but is susceptible to cobalt and nickel. The plant is propagated through seeds. Seedlings are first raised in the nursery and then transplanted in the field. The seed rate is about 125 g/ha for transplanting. Seeds start germinating 3 days after sowing and germination is over in 7-10 days. When 6-10 cm tall the seedlings are transplanted in the field at 40-60 cm spacing in rows. At the time of planting, 10-15 tonnes of compost or farm yard manure is to be applied. A medium fertilizer dose of 40:40:40 kg/ha of N, P_2O_5 and K_2O is

recommended for economic yield though good response has been received upto 120:100:100 kg/ha. Irrigation is required once a week when it is raised as a summer crop. The field should be kept weed free for the first 20-25 days, till the crop canopy completely covers the ground. Weeding is usually carried out once or twice.

The *O.gratissimum* can be harvested when the plant is in full bloom and lower leaves start turning yellowish. The crop comes to full bloom 9-12 weeks after planting. For high quality oil, only the flowering tops are harvested. 4-5 crops are obtained per year. In some areas it is possible to get four floral harvests. The first harvest is done when the plants are in full bloom and the subsequent ones after every 15-20 days. The last harvest comprises the whole plant. Floral harvests yield 3-4 tonnes of flowers and the final harvest of the whole plant is 13-15 tonnes of herb per hectare. While harvesting the whole herb, plants are cut not less than 15 cm from the ground for enabling regeneration of the crop. (Thomas *et al.,* Aromatic plants, 1998)

2.5. Traditional medicinal uses:

The whole plant and the essential oil have many applications in the traditional medicine, especially in India and Africa. Preparations from the whole plant are used as stomachic and in treating sunstroke, headache and influenza. The seeds have laxative properties and are prescribed against gonorrhea. The essential is applied against fever, inflammations of the throat, ears or eyes, stomach pain, diarrhea and skin diseases and it is being used as anti-biotic.

In Indonesia a tea is made from the leaves while in Thailand, the leaves are applied as a flavoring agent. In Indonesia the eugenol type of *O.gratissimum* is used in the ceremonial washing of corpses and is planted in graveyards. In India, *O.gratissimum* is being named as “Ram Tulasi” and widely used in religious ceremonies and rituals. (Orwa *et al.,* 2009)

In coastal area of Nigeria, it is used for the treatment of epilepsy, high fever and diarrhea. The plant is used to treat typhoid fever and diabetes. Also the essential oil of *O.gratissimum* possessed insecticidal, larvicidal, anti-bacterial, and anti-fungal properties.

In Brazil, it is called as alfavaca and used to treat for upper respiratory tract infection, diarrhea, headache, ophthalmic and skin diseases and pneumonia also to treat cough, fever, toothache and snake bite. (Silva *et al.,* 2005)

In Kenya and sub-urban Africa, the leaves of this, plant rubbed and sniffed as treatment for blocked nostrils and also used for abdominal pain, sore eyes, ear infection, and regulation of menstruation also as a cure for prolapse of rectum. (Lexa *et al.,* 2007)

2.6. Phytochemical studies:

Mohammed *et al.,* **1998** was reported the chemical composition of *O.gratissimum* essential oil obtained from Bangladesh, evaluated by GC and GC-MS analysis and the oil was classified as thymol type. The results represented about 97% of the whole oil and the main component was thymol (58.2%), followed by ϒ-terpinene (12.3%) and p-cymene (7.7%). The total content of phenol compounds was 59.5% and the monoterpene hydrocarbons represented 30.9%. The sesquiterpene hydrocarbons 2.8%, alcohols 2.8% and three oxides 1,8-cineole, caryophyllene oxide and humulene oxide II, and small quantities of a ketone, α-Thujone.

Keita S M *et al.,* **2000** was analyzed the composition of essential oil of different *Ocimum* species (*O.basilicum*, *O.gratissimum* and *O.suave*) in the Republic of guinea by GC and GC-MS. The oil showed the following major constituents, linalool (69%), eugenol (10%), t-α-bergamotene (3%) and thymol (2%) in *O.basilicum* whereas *O.gratissimum* contains thymol (46%), p-cymene (12%) and ϒ-

terpene + t-sabiene hydrate (17%). In the case of *O.suave*, showed the p-cymene (59%), α-thujene (10%), myrcene (7%), thymol (7%) respectively. The maximum oil yield from leaves from *O.basilicum* 1.8% v/w, when compared with *O.gratissimum* 1.0% v/w and *O.sauve* had 1.7% v/w respectively.

Vieira R F *et al.,* **2001** was studied the genetic relationship of *O.gratissimum* based on volatile oil constituents, flavonoids and RAPD markers. The major constituents in isolated oil was eugenol, thymol, geraniol and some flovones (xantomicrol, cirsimaritin). The accessions morphologically described as *O.gratissimum* var. *gratissimum* and *O.gratissimum* var. *macrophyllum* were found genetically more distant from the other two groups when analyzed by molecular markers. Cluster analysis of RAPD markers showed that three groups of distinction genetically and highly correlated (r=0:814) to volatile oil constituents.

Leopold *et al.,* **2003** was evaluated the chemo-taxonomical properties of essential oil of four different *Ocimum* species (*O.americanum, O.basilicum, O.gratissimum and O.sanctum*) from southern India by SPME/GC-FID, SPME/GC-MS and olfactoric methods. The essential oil of the whole plant of *O.americanum* shown rich in (E)-methyl cinnamate (72.05%), whereas the essential leaf oil of *O.gratissimum* comprises more account of eugenol (63.36%). Therefore, the following chemotypes can be attributed to the analysed *Ocimum gratissimum*, eugenol-type.

Maria G V S *et al.,* **2004** was used the different techniques (steam distillation, microwave distillation and supercritical CO_2 extraction) to isolate the volatile oil of *O.gratissimum* and evaluated by GC-MS analysis. The result indicated that the oil contain eugenol 54.0% and 1,8-cineole 21.6% as major compounds by steam distillation. The microwave distillation shown main constituent of eugenol 34.6% and

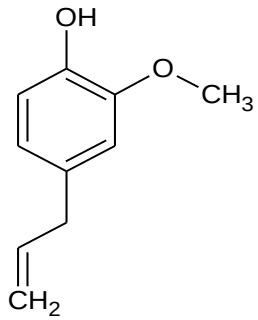
1,8-cineole 22.6% in the case of supercritical CO_2 extraction presented eugenol 73.1% and 1,8-cineole 5.6%.

Afolabi *et al.,* **2007** was investigated the phytochemical constituents of both methanolic and aqueous extract of *O.gratissimum* leaves. The extracts showed tannins, steroids, terpenoids, flavonoids and cardiac glycosides as main constituents.

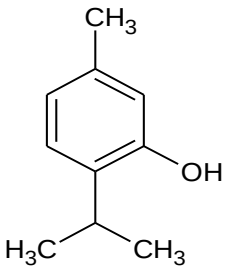
Adamu *et al.,* **2008** was reported the phytochemical constituent of O.gratissimum leaves by soxhlet apparatus extraction using water. The extract was showed such as tannins, saponins, cardiac glycosides, reducing sugars (free and combined), terpenes, flavanoids and alkaloids but not contain ketones and anthraquinone derivatives.

Idris S *et al.,* **2011** was researched on proximal and mineral composition of the leaves and stems of *O.gratissimum* aqueous extract. The report was showed the 82.60 ± 0.01% and 82.61 ± 0.11% of moisture content for leaves and stems and the ash content was 13.67 ± 0.13% and 13.67 ± 0.02% for leaves and stems. The crude protein was existed 3.33 ± 0.07% and 1.65 ± 0.02% in leaves and stems and the leaves and stems showed the crude lipid content was 8.50 ± 0.04% and 3.00 ± 0.15% respectively.

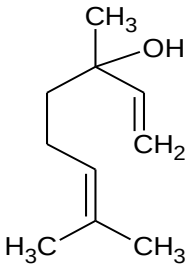
Rattanawat *et al.,* **2010** was analyzed the effects of soil amendments and metal uptake by *O.gratissimum* grown in cadmium/zinc-contaminated soil by the hydroponic study. The *O.gratissimum* accumulated the metals (cadmium and zinc) in roots more than shoots, and the metal concentration significantly increased ($p \leq 0.05$) with the increase of metal concentration in the solution. The highest cadmium accumulation was found in plants exposed to 5 mg/l of cadmium solution and zinc accumulation at 20 mg/l of zinc solution.



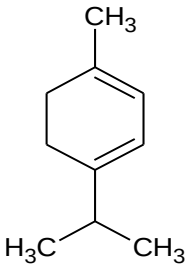
Eugenol



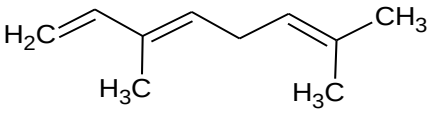
Thymol



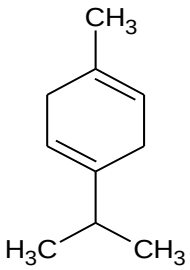
Linalool



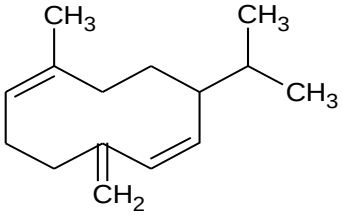
Alpha-terpene



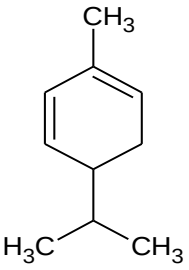
Trans-beta-ocimene



gamma-terpinene



Germacrene D



alpha-phellandrene

Fig. 3: structure of chemical compounds present in *O.gratissimum* oil

2.7. Pharmacological studies:

2.7.1. Anti-microbial activity:

Anand A K *et al.,* **2011** was investigated the anti-microbial activity of three *Ocimum* species (*O.basilicum, O.gratissimum* and *O.kilimandscharicum*) of volatile oil collected from Uttarkhand (India) by GC and GC-MS. Among these three species, *O.gratissimum* eugenol (53.89%) was the most abundant component followed by cis-ocimene (23.97%) and germacrene-D (10.36%). The oil of species tested against standard bacterial strains *S.aureus, E.faecalis, E.coli, P.aeruginosa* and yeast *C.albicans* by broth dilution method and the *O.gratissimum* oil was found to be more active against all tested micro-organisms, especially against *C.albicans* and gram positive bacteria.

Verma R S *et al.,* **2011** was analyzed the chemical composition and anti-bacterial activity of essential oil from two *Ocimum* genus (*O.gratissimum and O.kilimandscharicam*) grown in sub-tropical region of northern India during spring/summer cropping season by capillary GC-FID and GC-MS methods. The major component of the *O.gratissimum* oil reported, eugenol (63.7%), (Z)-β-ocimene (19.6%) and germacrene-D (7.3%). The anti-bacterial activity was tested against four pathogenic gram positive bacterial strains by disc diffusion assay and the *O.gratissimum* oil was found to be higher active against all tested strains when compared with *O.kilimandscharicam* oil. The maximum zone of inhibition was observed in *Staphylococcus aureus* (16.0 mm), followed by *Staphylococcus mutans* (14.0 mm), *Enterococcus faecalis* (10.0 mm) and *Staphylococcus epidermidis* (8.0 mm). The eugenol was demonstrated to have anti-bacterial property due to presence of eugenol in good percentage.

Ahonkhai *et al.,* **2009** was studied the anti-microbial activities of the volatile oils of *O.bacilicum* and *O.gratissimum* against some aerobic dental isolates (*Streptococcus viridians, Staphylococcus albus, Klebisiella pneumonia, Pseudomonas aeruginosa* and *Proteus vulgaris)* and evaluated on by twenty nine organisms using agar diffusion and agar dilution methods. In the susceptibility tests, the volatile oils of *O.basilicum* and *O.gratissimum* independently inhibited the growth of *Klebisiella pneumonia* at a con. of 0.51% in the agar; *Streptococcus viridians* and *Staphylococcus albus* at 1.10% and *Pseudomonas aeruginosa* at 10.0%. *Proteus vulgaris* was inhibited at 0.53% by the volatile oil of *O.gratissimum* and 0.67% by *O.basilicum*. Separate incorporation of the volatile oils into tooth pastes (2 and 5%), the volatiles oils showed anti-bacterial activities comparable to a commercial tooth paste (which contains *O.basilicum* 0.01% among others) against most resistant organisms. As components of mouth washes, the volatile oils completely inhibited the growth of organisms at a con. of 0.5%.

Erute M O *et al.,* **2008** was studied the different extracts (ethanolic, sterilized and un-sterilysed water) of three different species (*Ocimum gratissimum, Acalypha wilkesiana* and *Acalypha macrostachya)* and assessed for inhibitory effect on the radial growth of *Cercospora purpurea*. The ethanolic extracts of the three plants caused total inhibition of *C. purpurea* and crude extract of sterilized and un-sterilized water significantly reduced the radial growth of the fungus. However, the fungistatic effect of *O.gratissimum* was highest followed by *A.macrostachya* and then *A.wilkesiana* respectively.

Mbata *et al.,* **2008** was investigated the anti-bacterial effect of ethanolic extract of *O.gratissimum* against *Listeria monocytogenes* serotype 4a by agar well diffusion and tube dilution methods. The bacterium was grown at 37^{o} C in a

chemically defined or a complex medium, containing essential oil obtained from *O.gratissimum*. The essential oil progressively inhibited the bacterial growth at con. from 20 to 250 μg/ml. The bacteria cultivated on chemically defined medium was more sensitive to essential oil at concentration of 50, 62.5 and 100 μg/ml in relation to those cultivated in complex medium at 37^{o} C. The organism was evaluated by agar well diffusion method and the zone of inhibition showed at 25 mm.

Lexa G M *et al.,* **2007** was reported the chemical composition and anti-microbial activity of essential oil of *O.gratissimum* growing in eastern Kenya, by GC-MS evaluation. The oil was dominated by monoterpens which accounted for 92.48%. Eugenol (68.8%) was the major constituent of monoterpenes and methyl eugenol (13.21%), cis-ocimene (7.47%), trans-ocimene (0.94%), β-pinene (1.10%), camphor (0.95%) as other constituents. The oil contained germacrene D (4.25%), trans-caryophyllene (1.69%), α-farnesene (0.85%) and β-bisabolene (0.74%) as sesquiterpenes. The anti-microbial activity of the essential oil was evaluated against both gram-positive (*S.aureus, Bacillus* spp.), gram-negative (*E.coli, P.aeruginosae, S.typhi, K.pneumoniae, P.mirabilis*) bacteria and a pathogenic fungus (*C.albicans*) and was found to be active against all microbes, although the concentration of oil was generally higher than the standard anti-biotic chloramphenicol.

Silva *et al.*, **2005** was analyzed the different extracts (ethanolic extract, ethyl acetate extract, hexane extract and chloroform extract) of *O.gratissimum* and the essential oil of its leaves by clevenger type apparatus and their anti-bacterial activity was screened by agar dilution technique towards dermatophytes (*M.canis, T.mentagrophytes, T.rubrum and M.gypseum*). The analysis revealed that chloroform and hexane fractions, essential oil and eugenol of *O.gratissimum* have antifungal activity against all dermatophytes while ethyl acetate fraction was active against

M.canis and *T.mentagrophytes*. The report also suggested that the hexane extract (100% inhibition at 125 µg/ml) and eugenol (80% inhibition at 125 µg/ml) have most active.

Nakamura *et al.,* **2004** was carried out the effects of *O.gratissimum* essential oil against *Candida* fungus (*C.albicans, C.krusei, C.parapsilosis, and C.tropicalis*) by determination of MIC and time-kill curves. The analysis of ultrastructure of the yeast cells revealed changes in the cell wall and in the morphology of some sub-cellular organelles. The result was demonstrated that the essential oil of *O.gratissimum* showing fungicidal activity against all of the *Candida* species.

Iwalokun *et al.,* **2003** was performed the effects of essential oil of *O.gratissimum* at sub-inhibitory con. on virulent and multi-drug resistant *Shigella* strains from Lagos (Nigeria). The essential oil caused significant decreases ($P<0.01$) in extracellular protease activity, o-lipopolysaccharide rhamnose content and incidence of invasiveness mediated as kerato-conjunctivitis in guinea pig and showed greater anti-virulent effect at 1.0 mg/ml. The anti-biotic susceptibility test was revealed that the essential oil of *O.gratissimum* reduced the MICs of anti-biotics to which *Shigellae* showed resistance by 9.8–53.1% and fluoroquinolones by 18.2–45.5%. The results of this study strongly suggested the inhibition of extracellular protease and expression of O-LPS rhamnose in *Shigellae* by *O.gratissimum* essential oil.

Nakamura C V *et al.,* **1999** was analyzed the anti-bacterial activity of essential oil of *O.gratissimum* fresh leaves by GC-MS analysis. The inhibition zones of essential oil was ranging from 13 to 25 mm against six strains of gram-positive or gram-negative bacteria (*Proteus mirabilis, Klebsiella sp, Escherichia coli, Salmonella*

enteritidis, Staphylococcus aereus and *Shigella flexineri*) using the diffusion technique on solid media.

Janssen *et al.,* **1989** was screened the anti-bacterial activity of essential oils of four *Ocimum* species (*O.canum, O.gratissimum, O.trichodon and O.urticifolium*) grown in Rwanda against different micro-organisms (*E.coli, B.subtilis, S.aureus* and *T.mentagrophytes var. interdigitale)* by dilution technique and was found to be anti-microbially active.

2.7.2. Analgesic activity:

Vikram *et al.,* **2011** was studied the anti-nociceptive activity of methanolic extract of *O.gratissimum* and acetic acid was used to study this effect to induce the abdominal constriction by hot-plate method in mice. The extract was demonstrated a significant ($P<0.05$) anti-nociceptipe activity at all the doses (50,100 and 200 mg/kg body weight i.p.) tested group and standard group (piroxicam 20 mg/kg) compared to control normal saline. The activity resides more at the highest dose 200 mg/kg body weight i.p. that was found to have the highest percentage (92.5%) of inhibition of the abdominal constriction induced by acetic acid in mice.

Aziba *et al.,* **1999** was screened the analgesic activity of aqueous extract of *O.gratissimum* in mice and the significant effect was recorded at 10 mg/kg and 23.2 mg/kg when compared with reference drug Panadol 125 mg/ml. The results was indicated the presence of analgesic property of the extract.

2.7.3. Anti-cancer activity:

Makker P N *et al.,* **2007** was investigated the anti-cancer activity of aqueous extract of *O.gratissimum* on human breast cancer utilizing *in vitro* and *in vivo*

methodologies. The aqueous extract of leaves *O.gratissimum* inhibits proliferation, migration, anchorage independent growth, 3D growth, morphogenesis and induction of COX-2 protein in breast cancer cells. A comparative analysis with eugenol, apigenin and ursolic acid showed that the inhibitory effects on chemotaxis and 3D morphogenesis of breast cancer cells specific to the *O.gratissimum* extract.

2.7.4. Anti-convulsive activity:

Galindo L A *et al.,* **2010** was evaluated the anti-convulsive effects of *O.gratissimum* essential oil (contain eugenol, 1,8-cineole and trans-caryophyllene) by open-field and rota-rod tests (separately or mixed in the same proportion) against, convulsions induced by pentylenetetrazole (60 mg/kg, subcutaneously) or maximal electroshock (50 mA, 0.11 s) and in sodium pentobarbital (45 mg/kg, intra-peritoneally) induced sleeping time. The compounds, increase sleeping time duration when in association than separate mode. The result was indicated that the major compounds not always responsible for a biological effect observed in medicinal plant preparations.

2.7.5. Anti-oxidant activity:

Hakkim L *et al.,* **2008** was investigated the ethanolic extract of some *Ocimum* species (*O.gratissimum, O.americanum, O.minimum, O.citriodorum, O.kilimandscharicam, O.grandiflorum, O.lamiifolium and O.selloi*). The quantitative analysis of phenolic constituents was determined using HPLC and the total phenolic content was estimated using Folin-Ciocalteu reagent. The anti-oxidant activity was assessed using iron (III) reduction, β-carotene-linoleic acid bleaching, 1,1-diphenyl-2-picrylhydrazyl and superoxide anion free radical scavenging assays. The extracts of

Ocimum species exhibited anti-oxidant activity but it was not as potent as butylated hydroxyl anisole.

Trevisan *et al.,* **2006** was evaluated the anti-oxidant capacity of essential oils of five *Ocimum* species (*O.basilicum* var. *purpurascens, O.basilicum, O.gratissimum, O.micranthum, and O.tenuiflorum*) obtained by steam hydro-distillation using a HPLC-based hypoxanthine/xanthine oxidase and the DPPH assays. The *O.gratissimum* was produced 3.5% of oil and shown strong anti-oxidant capacity but the greater was shown by *O.tenuiflorum* (IC_{50} = 0.46 µl/ml).

Leal P F *et al.,* **2006** was analyzed the extract of *O.gratissimum* obtained by supercritical fluid extraction method by GC-FID and the antioxidant factor was assessed using the coupled reaction of β-carotene and linolenic acid. The extracts global yields varied from 0.91 to 1.79% (dry basis), and the antioxidant activity was varied from 62 to 84% (eugenol 35 to 60% and b-selinene remained approximately constant from 11.5 to 14.1%) compared with the control β-carotene.

2.7.6. Anti-inflammatory activity:

Mahapatra *et al.,* **2009** was studied the effect of aqueous extract of *O.gratissimum* on immune functions and immune responses in nicotine-induced (10 mM) macrophages. Release of Th1 cytokines (TNF-α, IL-12) and Th2 cytokines (IL-10, TGF-β) was measured by ELISA, and the expression of these cytokines at mRNA level was analyzed by real-time PCR. The administration of aqueous extract and ascorbic acid increased the decreased adherence, chemotaxis, phagocytosis, and intracellular killing of bacteria in nicotine-treated macrophages. The aqueous extract and ascorbic acid was found to protect the murine peritoneal macrophages through

down regulation of Th1 cytokines in nicotine-treated macrophages with concurrent activation of Th2 responses. These findings strongly enhanced our understanding of the molecular mechanism leading to nicotine-induced suppression of immune functions and provide additional rationale for application of anti-inflammatory therapeutic approaches by *O.gratissimum* and ascorbic acid for different inflammatory disease prevention and treatment during nicotine toxicity.

2.7.7. Hypoglycemic activity:

Aguiyi *et al.,* **2000** was eveluated the hypoglycaemic effect of the methanolic extract of *O.gratissimum* leaves in normal and alloxan-induced diabetic rats. The intra-peritoneal injection of the extract (400 mg/kg) significantly reduced plasma levels both in normal and diabetic rats by 56 and 68%, respectively.

2.7.8. Larvicidal activity:

Rajamma *et al.,* **2011** was performed the larvicidal activity of essential oil of different *Ocimum* species (*O.santum, O.basilicum and O.gratissimum*) and their different extracts against larvae of *Culex quinquefasciatus* by TLC analysis. It revealed that all the three species have similar components and results showed the presence of steroids and triterpenoids. The *O.basilicum* was found to be more active in larvicidal activity than the other two species.

2.7.9. Hepato-protective activity:

Adejoke *et al.,* **2011** was studied the ethanolic extract of *O.gratissimum* causes dose dependent hepatotoxicity in Streptozotocin-induced diabetic wistar rats. The extract was contain carbohydrates, reducing sugars, lipids, flavonoids, alkaloids,

steroids, glycosides,tannins, resins and the results shown reduction in blood sugar, increase in biochemical and histologic markers of hepatic injury.

Arhoghro *et al.,* **2009** was tested the aqueous extract of scent leaf (*O.gratissimum*) on CCl_4 induced liver damage in albino wister rats. The results of the study indicated that *O.gratissimum* might be an effective plant hepato-protector in the diet of patients with hepatopathies.

2.7.10. Acute and sub-chronic toxicity:

Orafidiya *et al.,* **2004** was performed the research on acute toxicity and the sub-chronic toxicity of the essential oil of *Ocimum gratissimum* and the data analyses of blood biochemical, haematological and histo-pathological findings showed significant differences between control and treated groups and revealed that *Ocimum* oil have capable of invoking an inflammatory response that transits from acute to chronic on persistent administration.

2.7.11. Anti-diarrhoeal activity:

Ezekwesili *et al.,* **2004** was evaluated the anti-diarrhoeal activity of aqueous extract of *O.gratissimum* in rats by testing in various doses (25, 50 & 100 mg/kg body weight) displayed remarkable anti-diarrhoeal activity that was evidenced by the reduction in the rate of defecation and consistency of faeces. The protective role of *O.gratissimum* extract at 100 mg/kg body weight was comparable to that of the reference drug, diphenoxylate (50 mg/kg body weight).

2.7.12. Blood coagulation factor:

Edemeka *et al.,* **2000** Was studied the effects of aqueous and methanol extracts of *O.gratissimum* leaves on the prothrombin time, activated partial thromboplastin time of normal plasma and Factor VIII-deficient plasma. A test

mixture with methanol extract significantly shortened the activated partial thromboplastin time of normal and Factor VIII-deficient plasma samples and study reveals that the biological activity of extract contains unidentified constituents that promote blood coagulation.

AIM AND

PLAN OF WORK

3. AIM AND PLAN OF WORK

3.1. Aim:

The present study was designed to find out the chemical composition of the essential oil of *O.gratissimum* Linn., grown in Tamilnadu by GC-MS method. Further isolation chemical constituents from aqueous extract of *O.gratissimum* Linn., and identified by IR, NMR and Mass spectroscopy methods. The anti-microbial activity of volatile oil and aqueous extract was checked against some micro-organisms. To the best of our knowledge, no such comparison studies have been reported earlier in the literature.

3.2. Plan of work:

- Collection of the fresh leaves of *O.gratissimum* plant.
- Authentication of *O.gratissimum* leaves.
- Isolation of essential oils from *O.gratissimum* by using Clevenger apparatus with water as solvent.
- Determination of organoleptic characters, solubility and percentage yield of volatile oils.
- Measurement of following Physiochemical constants of essential oil of *O.gratissimum* leaves.

  a. Specific gravity

  b. Optical rotation

  c. Refractive index

- Analysis of chemical composition of essential oil of *O.gratissimum* leaves by GC-MS analysis.
- Preparation of aqueous extract of *O.gratissimum* by different methods.
- Preliminary phytochemical studies of *O.gratissimum*
- Quantitative estimation of phytochemicals.
- Study of physico-chemical constant of *O.gratissimum* leaves aqueous extract.
- Qualitative analysis of inorganic compounds in *O.gratissimum* leaves.
- Quantitative analysis of inorganic compounds in *O.gratissimum* leaves
- Column chromatography of *O.gratissimum* aqueous extract.
- Identification of isolated compound by IR, NMR and Mass spectroscopy methods.
- Anti-microbial activity of volatile oil and aqueous extract of *O.grattissimum* leaves.

EXPERIMENTAL

4. EXPERIMENTAL

4.1. Materials

4.1.1. Plant raw material and Chemicals:

1. *Ocimum gratissimum* leaves
2. Distilled water
3. Anhydrous calcium chloride
4. Cotton

4.1.2. Instruments Used:

Table 1: Instruments/Apparatus

S.No.	Instruments	Specification/Make
1	Clevenger apparatus	Borosil glass work Ltd., Mumbai.
2	Rotary shaker	Remi Equipments., Chennai.
3	Soxhlet apparatus	Borosil glass work Ltd., Mumbai.
4	Microwave oven	CMS 19 Godrej Ltd., Mumbai.
5	Weighing Balance	SHIMADZU AUX 220., Japan.
6	Polarimeter	Sipcon industry Pvt.Ltd., Mumbai.
7	Refractometer	Sipcon industry Pvt.Ltd., Mumbai.
8	GC-MS	PerkinElmer Clarus 50.
9	IR	Perkin-Elmer.
10	NMR	Bruker-NMR 500MHZ.
11	MASS	JEOL GC mate.

4.1.3. Micro-organisms:

Three bacterial strains (*Basillus cereus, Basillus subtilus, Staphylococcus*) and three fungal organisms (*Aspergillus flavus, Candida albicans, Aspergillus niger)* were collected from Microbial Resources Division, Kings Institute of Preventive Medicine, Guindy, Chennai. The agar medium and sabouraud's dextrose agar medium was purchased from HI-Media Laboratories Ltd., Mumbai, India.

4.2. Isolation of volatile oil

4.2.1. Collection of *Ocimum gratisimum* leaves:

The leaves of *Ocimum gratissimum* (Family: Lamiaceae) were collected in the month of July 2011 in Ooty, Tamilnadu and it was authenticated by Dr. G.V.S. Murthy, Scientist "F", Botanical Survey of India, Coimbatore. The herbarium specimen of the plant was preserved at Department of Pharmacognosy, Adhiparasakthi College of Pharmacy, Melmaruvathur.

4.2.2. Isolation of the essential oil:

Fresh leaves of *O.gratissimum* were subjected to water distillation for 4 h using a Clevenger apparatus to produce the essential oil. The oil was dried over anhydrous $CaCl_2$ and store in sealed vials at low temperature before analysis. The percentage yield of essential oil of each variety was estimated by using the following equation and reported.

$$RHE\ (\%) = (mHE/mS) \times 100$$

Where, RHE = essential oil yield (%), mHE = essential oil mass (g), and

mS = dry leaves mass (g).

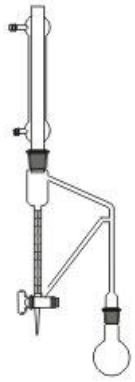


Fig. 4 : Clevenger apparatus

4.2.3. Physical and chemical evaluation of volatile oil

4.2.3.1. Determination of organoleptic properties:

The volatile oil was placed in a transparent bottle over a white back ground and the color and clarity were observed and the characteristic odor was determined by sniffing and to determined its characteristic feel to the touch, it was rubbed between fingers.

4.2.3.2. Determination of solubility:

The solubility of the *O.gratissimum* volatile oil was determined by mixing increment volumes of the volatile oil in specified volumes of the following solvents: water, chloroform, alcohol, anhydrous ether and petroleum ether.

4.2.3.3. Determination of specific gravity:

Specific gravity is an important criterion of the quality and purity of volatile oils. The actual weight or the tare of a vial or was determined accurately using a SHIMADZU AUX 220 balance. The vials filled with water and weighed. The procedure was repeated using the *O.gratissimum* volatile oil in place of water, and the specific gravity of the oil was expressed as the ratio of the weight

of the volume of oil to that of an equal volume of pure water when both are determined at 25^{o} C.

4.2.3.4. Determination of optical rotation:

Both the degree of rotation and its direction are important criteria of purity. The extent of optical activity of oil was determined by a Polarimeter by which measured the degree of rotation. The zero point of the Polarimeter was adjusted and determined. The previously cleaned and dried Polarimeter tube was filled with 10% alcoholic solution of the volatile oil. The analyzer was rotated until equal illumination of light of the two halves of the visual field was achieved. The angle of rotation was determined and the specific rotation was calculated.

$$[\alpha] = t/\lambda = 100\ \alpha\ /lcd$$

Where: $[\alpha]$ = Specific rotation at, t = temperature, λ = observed rotation in degrees,

c = concentration, l = path length in dm, λ = wavelength of light used in nm, d = specific gravity of the oil.

4.2.3.5. Determination of refractive index:

The index of refraction is a physical constant frequently made use of in determination of the identity and purity of volatile oils. The test plate was attached to the refracting prism of the Sipcon refractometer provided with the test plate, by moistening the test plate with the liquid and pressing it against the refractive prism. The light was focused on the test plate. The instrument was adjusted until the borderline of the critical angle coincides with the cross hairs in the telescope, and the reading of the refractive index was taken. The test plate was removed, cleaned, and 2-3

drops of *O.gratissimum* volatile oil was placed on the prism and the prism was clamped together firmly. Fixed the light source, so that the light was reflected through the prism and the instrument adjusted until the borderline between the light and dark halves of the field of view exactly coincides with the cross hairs of the telescope. The refractive index was then read and reported. (Ashutosh Kar., 2007)

4.2.3.6. GC-MS analysis of the *O.gratissimum* volatile oil:

The volatile oil of *O.gratissimum* was chemically characterized using Clarus 50 Perkin Elmer GC-MS.

The identification of the compound in the oil was based on the retention time and peak area. The relative percentage of the oil constituents was calculated and the percentage of compound was calculated as the ratio of the peak area to the total chromatographic area. The GC-MS peaks were identified by comparison with several data reported by other researchers and the profiles from the NIST'05 libraries.

Instrument Details:

Make: PerkinElmer Clarus 50

Column Type: Capillary Column Elite-5 ms (5% Phenyl 95% dimethylpolysiloxane)

Column length: 30 m

Column id: 250 µm

GC Conditions:

Oven Program: 50º C@ 5º C/min to 150º C (5 min) @ 10º C/min to 250º C

Injector temp. : 250º C

Carrier gas: He @ 1 ml/min

Split ratio 1:40

MS Conditions:

Mass Range: 40-450 amu

Electron energy: 70 ev

Source and Inlet line temperature: 200º C

Scan mode: Full Scan

Library used for matching: NIST'05

4.3. Preparation of aqueous extract:

4.3.1. By Mechanical method:

10 g of air dried powder of leaves weighed and placed in 100 ml of distilled water in a conical flask and then kept in a rotary shaker at 190-240 rpm for 24 h. It was filtered with the help of muslin cloth and centrifuged for 15 min. The supernatant was collected and the solvent was evaporated by using rotary vacuum evaporator. It was stored in a refrigerator at 10° C in air tight bottles for further studies.

4.3.2. By Soxhlet apparatus:

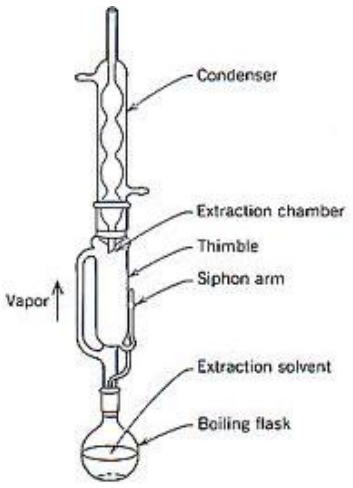


Fig. 5 : Soxhlet apparatus

The 50 g of leaves of *O.gratissimum* were washed with distilled water, air dried for five days and ground into fine powder using pestle and mortar. The powder was extracted by soxhlet apparatus with water for 10 h at 100^{o} C. The extract was then concentrated in vacuo using a rotary evaporator and stored at 4^{o} C until used. (Adamu M *et al.*, 2009)

4.3.3. By Microwave oven method:

About 100 g of dried *O.gratissimum* leaves were cut and grinded to small size, then mixed with water in suitable ratio. The suspension was radiated in microwave oven at regular intervals (one minute radiation and two minutes off) to keep temperature not rise above 80^{o} C. The infusion was let to cool down to room temperature, filtered and stored in refrigerator at 4^{o} C for further studies. (Pham T Q *et al.*, 2006)

4.4. Preliminary phytochemical screening:

4.4.1. Test for alkaloids (Mayer's test):

About 0.5 ml of extracts were separately treated with few drops of 1 ml 2 N HCl and filtered. (Mayer's reagent: 1.36 g of $HgCl_2$ was dissolved in 60 ml distilled water and 5 g of KI was dissolved in 10 ml of distilled water. The two solutions were mixed and made up to 100 ml of distilled water). Few drops of reagent were added to 1.0 ml of acidic aqueous solution of samples. Formation of white or pale precipitate indicated the presence of alkaloids.

4.4.2. Test for glycosides:

A small amount of alcoholic extract of sample was dissolved in 1.0 ml of water and then aqueous solution of NaOH was added. Formation of a yellow colour indicated the presence of glycosides.

4.4.3. Test for flavanoids:

In a test tube containing 0.5 ml of extracts of the sample, added 5-10 drops of dil. HCl and a small piece of Mg and the solution was boiled for few minutes. In the presence of flavanoids, the reddish pink or dirty brown colour was produced.

4.4.4. Test for reducing sugars:

A known quantity of extract was dissolved in 5 ml of distilled water and filtered. The filtrate was boiled with drops of Fehling's solution A and B for few minutes. An orange red precipitate was indicated the presence of reducing sugars.

4.4.5. Test for tannins (Lead acetate test):

In a test tube containing about 5.0 ml of an extract, added a few drops of 10% solution of lead acetate and produced formation of white colour precipitate that indicated the presence of tannins.

4.4.6. Test for anthraquinones:

The extract was boiled with 10% HCl for few minutes in a water bath then it was filtered and allowed to cool. The equal volume of $CHCl_3$ was added to filtrate followed by few drops of 10% NH_3 solution to the mixture and heated. Formation of rose-pink colour indicated the presence of anthraquinones.

4.4.7. Test for saponins:

The extract was diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 min. One cm length of foam was formed indicating the presence of saponins. (Kokate C K., 2007)

4.5. Quantitative estimation of the phytochemicals:

4.5.1. Determination of total phenols

To determine the total phenols, 5 g of the plant sample was weighed into a 250 ml titration flask and 100 ml n-hexane was added twice for 4 h; the filtrate was

discarded for fat free sample preparation. Then, 50 ml diethyl ether was added twice, was heated for 15 min, cooled up to room temperature and filtered into a separating funnel. About 50 ml of the 10% NaOH solution was added twice and shook well each time to separate the aqueous layer from the organic layer. It was washed three times with 25 ml de-ionized water. The total aqueous layer was acidified up to pH 4.0 by adding 10% HCl solution and 50 ml dichloro methane twice to acidify the aqueous layer in the separating flask. Consequently, the organic layer was collected, dried and then weighed.

4.5.2. Determination of flavonoids:

To determine flavonoids, 5 g of plant sample was weighed in a 250 ml titration flask, and 100 ml of the 80% aqueous methanol was added at room temperature and shaken for 4 h in an electric shaker. The entire solution was filtered through Whatman filter paper no. 42 (125 mm) and again, this process was repeated. The filtrate as a whole was later transferred into a crucible and evaporated to dryness over a water bath and weighed.

4.5.3. Determination of saponins:

For the saponins determination, 5 g of plant sample was weighed and dispersed in 100 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 h with continuous stirring at about 55° C. The filtrate and the residue was re-extracted with another 100 ml of 20% ethanol. The combined extract was reduced to 40 ml over water bath at about 90° C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and about 30 ml of n-butanol was added. The combined n-butanol extract was washed twice with 10 ml of 5 % aqueous sodium chloride. The

remaining solution was heated in a water bath. After evaporation, the sample was dried in the oven to a constant weight and calculated. (Iqbal H *et al*., 2011)

4.5.4. Determination of alkaloids:

About 5g of the sample was weighed into a 250 ml beaker then added 200 ml of 10% acetic acid in ethanol, covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate collected and washed with dilute ammonium hydroxide and then filtered. The residue of the alkaloid was dried and weighed. (Ladipo M K *et al.,* 2010)

4.5.5. Determination of reducing and non-reducing sugars:

Reducing and non-reducing sugar part of the plant leaves were estimated by titrimetric method using Benedict's quantitative reagent. The reagent was standardized using glucose stock solution (2 mg/ml). Sample stock solution was prepared by weighing 5 g of powder and macerated for 24 h with 100 ml distilled water for reducing sugar while for non-reducing sugar sample was boiled for 30 min with 5 ml of 0.1 N HCl on water bath with 25 ml of distilled water. 5 ml of each sample solution was then treated with fresh Benedict's reagent drop wise from the burette in warm condition until blue color changes to whitish. From the burette volume amount of sugar was calculated using formula 5 ml BR = 5 mg sugar. By calculation using dilution factor the reducing, non-reducing and total sugar of leaves were determined. (Ganpati K S *et al.,* 2011)

4.5.6. Determination of Tannins:

25 ml of infusion are measured into 1 l of conical flask, then 25 ml of indigo solution and 750 ml of distilled deionized water added. 0.1 N aqueous solution of

$KMnO_4$ was used for titration until blue colored solution changes to green color. Then few drops at time until solution becomes to golden yellow are added. Standard solution of indigo carmine was dissolved in 500 ml of distilled deionized water by heating, after cooling 50 ml of 95-97% H_2SO_4 was added. The solution was diluted to one liter and filtered. The blank tests by titration of mixture of 25 ml of indigo carmine solution and 750 ml of distilled deionized water were carried out.

Calculation:

The Tannin content in the sample was calculated,

$T\% = (V\text{-}V_o) \times 0.004157 \times 250 \times 100/g \times 100$

V = Volume of 0.1 N aqueous solution of $KMnO_4$ for the titration of the sample,

V_o = Volume of 0.1 N aqueous solution of $KMnO_4$ for the titration of the blank sample,

0.004157 = Tannins equivalents in 1 ml of 0.1 N aqueous solution of $KMnO_4$,

g = Mass of the sample taken for analysis, 250 = Volume of volumetric flask,

100 = Percent. **(**Attanassova M *et al.*, 2009)

4.6. Physico-chemical constant of aqueous extract of *O.gratissimum* leaves:

4.6.1 Estimation of total ash:

One gram of sample was taken in silica crucible and heated over a low bunsen flame to volatilize as much of the organic material as possible. The crucible was then transferred to a temperature controlled muffle furnace. The temperature was maintained at about 300º C for 5-7 h. It was removed from the muffle furnace and allowed to cool. After cooling, the weight of the ash was noted down.

4.6.2. Estimation of sulphated ash:

The silica or platinum crucible was heated to redness for 10 min, allow to cool in a desicator and weighed. Accurately weighed 1-2 g of substance into the crucible, ignited gently at first, until the substances is thoroughly charred. Cooled, moisten the residue with 1 ml of H_2SO_4, and heated gently until white fumes were no longer evolved and ignited at $800^o \pm 25^0$ C until all black particles have disappeared conduct the ignition in a place protected from air currents. Allowed the crucible to cool, added few drops of H_2SO_4 and heated. Ignited as before, allowed to cool and weighed.

4.6.3. Estimation of acid insoluble ash:

The total ash obtained was heated with 25 ml of 2 N HCl for 5 min and collected the insoluble matter on an ashless filter paper, washed with hot water, ignited and weighed. Calculated the percentage of acid insoluble ash with reference to the air dried sample.

4.6.4. Estimation of water soluble ash:

Boiled the ash for 5 min with 25 ml of water, collected the insoluble matter in a ashless filter paper, washed with hot water and ignited for 15 min at temperature not exceeding 450^o C. substracted the weight of the insoluble matter from the weight of the ash, the difference in the weight represents the water soluble ash. The percentage yield of water soluble ash was calculated with reference to the air dried sample.

4.7. Qualitative identification of inorganic elements:

To the ash of the drug material added 50% v/v HCl or 50% v/v HNO_3 to ash kept for one hour or longer. Filtered and the following test were performed using the filtrate.

4.7.1. Test for calcium:

(a) One drop dilute ammonium hydroxide and saturated ammonium oxalate solution added to the 10 ml of filtrate. Calcium oxalate was formed in white color precipitate, which was soluble in HCl but insoluble in acetic acid.

(b) With the solution of ammonium carbonate gave white precipitate which was insoluble in ammonium chloride solution.

4.7.2. Test for iron:

(a) Few drops of 2% potassium ferrocyanide were added to 5 ml of filtrate and it produced the dark blue colour.

(b) To 5 ml of filtrate, added few drops 5% ammonium thiocyanate solution and it showed blood red colour.

4.7.3. Test for sulphate:

(a) White crystalline barium sulphate precipitate was appeared when addition of few drops of 5% barium chloride to 5 ml filtrate, which was insoluble in HCl.

(b) With lead acetate solution gave the white colour precipitate and which was soluble in sodium hydroxide.

4.7.4. Test for phosphate:

To 5 ml of filtrate, added few drops ammonium molybdate solution and heated for 10 min and cooled, yellow crystalline precipitate of ammonium phosphomolybdate was observed.

4.7.5. Test for magnesium:

White colour precipitate was produced with ammonium carbonate solution but not with ammonium chloride solution.

4.7.6. Test for sodium:

(a) Flame test: Prepared thick paste of ash of the drug with conc. HCl and taken on the platinum wire loop and introduce in bunsen flame which produced the golden yellow flame.

(b) To 10 ml ash filtrate, added 2 ml of potassium pyroanthllollate and it gave the white colour precipitate.

4.7.7. Test for potassium:

(a) Few drops sodium cobalt nitrite solution was added to 2-3 ml of filtrate and it showed yellow precipitate of potassium cobalt nitrite.

(b) Flame test: Violet colour was observed to the flame.

4.7.8. Test for carbonate:

(a) Reddish brown precipitate was produced with mercuric chloride solution.

(b) White precipitate was formed with the solution of magnesium sulphate.

4.7.9. Test for nitrates:

(a) Red fumes were liberated when warmed with sulphuric acid and copper.

(b) With solution of ferrous sulphate gave no brown colour but if sulphuric acid was added (slowly from side of the test tube), produced brown colour at the junction of the two liquids.

4.8. Quantitative estimation of inorganic elements in *O.gratissimum* leaves:

4.8.1. Estimation of Calcium:

EDTA titrimetric method was used for determination of Calcium. 10 ml of sample was taken in a 100 ml titration flask and 10 ml of distilled water was added to it. Added 0.4 ml of 2 N NaOH to the diluted sample with the help of micropipette, followed by addition of 0.04 g of murexide indicator. The color of the solution turned pink. The sample was titrated against 0.1 M EDTA solution with constant stirring till the appearance of the light purple color as end point. Blank determination was carried out by repeating the same procedure using distilled water instead of sample. The concentration of sample was determined using the following formula. Triplicate reading were taken for sample and reported as mean of three readings.

Calculation:

Ca (mg/ml) = A.B.400.8/Volume of sample (ml)

Where A = Volume of titrant used (EDTA in ml)

B = Volume of $CaCO_3$ equivalent to 1.00 ml **(**Farhat A.K *et al.,* 2011)

4.8.2. Estimation of Magnesium:

The concentration of Magnesium in the sample is actually equal to the "difference between Calcium and total hardness as $CaCO_3$, if interfering metals are present in non-interfering concentrations in the Calcium titration and suitable inhibitors are used in the hardness titration. The following equation was used to calculate Magnesium from the values of total hardness and Calcium in the samples.

The method was repeated as triplicate and data was recorded as mean value of triplicate.

Calculation:

Magnesium (mg/ml) = (Total hardness – 2.5) Calcium 0.243.

(Farhat A.K *et al.*, 2011)

4.8.3. Estimation of Sodium and Potassium:

Mixed standard solution of Na^+ and K^+ were prepared by dissolving 254.2 mg of NaCl and 190.6 mg of KCl in 1 litre of methanol, so it gives 100 ppm (4.35 mM/l) of Na^+ and 100 ppm (2.558 mM/l) of K^+ solution. Similarly mixed standard solution of above elements were also prepared using double distilled water as a solvent in a same concentration as that of methanol.

Measurement procedure:

Sample of *O.gratissimum* leaves was analysed for element detection using mixed standard and double distilled water as a reference. For methanolic extract of plant leaves, mixed standards prepared in methanol was used and for water extract mixed standards prepared from double distilled water was used. Here instrument was given concentration of elements in mM/l. Calculated the amount of elements present in terms of mg/100 g fresh *O.gratissimum* leaves.

Calculation:

ppm = m.mole of ion x Molecular weight

mg of ion present in 10 gm of dry wheat grass powder =ppm x 1000/100000

(obtained from 100 gm fresh leaves of *O.gratissimum*) (Shah K.V *et al.*, 2011)

4.9. Column chromatography for aqueous extract of *O.gratissimum*

The column was packed with slurry of silica gel (mesh size, 60-120) with chloroform and then dried aqueous extract (4 g) of *O.gratissimum* prepared by microwave oven was first dissolved in water, carefully applied by pipette at the top of prepared column. Immediately after application of sample, a gradient of chloroform and methanol (mobile phase) was used as eluant to collect fractions of aqueous extract of *O.gratissimum.* The column was run with a gradient of chloroform : methanol (98:2, 95:5, 90:10, 80:20, 70:30, 50:50, 30:70, 20:80, 10:90, 5:95, 2:98) finally 100% methanol and their fractions were collected. Thereafter, from all the collected fractions solvent was removed by evaporation at room temperature. After evaporation of solvent from the fractions colorless crystals were isolated. The crystals of fractions were first separately treated with Petroleum ether and then filtered. The crystalline residues were then retreated with chloroform and were recovered after filtration. The identity of crystals was confirmed by spectroscopic analysis. (Dimpy D B *et al.,* 2011)

4.10. Screening of anti-microbial activity:

4.10.1. Anti-microbial activity of *O.gratissimum* leaves oil:

The leaf oil was tested for antimicrobial activity by disc diffusion method. The oil was diluted in DMSO in the ratio of 1: 10 and sterilized by filtering through 0.45 μm millipore filter. A final inoculum of 100 μl suspension containing 10^8 CFU/ ml of each bacterium and fungus was used. Nutrient agar (anti bacterial activity) and sabouraud's dextrose agar medium (antifungal activity) was prepared and sterilized by an autoclave (121º C and 15 Ibs for 20 min) and transferred to previously sterilized petridishes (9 cm in diameter). After solidification, petriplates were inoculated with bacterial organisms, in sterile nutrient agar medium at 45º C, fungal organisms in sterile sabouraud's dextrose agar medium at 45º C in aseptic condition. Sterile

Whatmann filter paper discs (previously sterilized in UV lamp) were impregnated with crude extracts at concentrations of 25, 50, 100 µl/disc were placed in the organism-impregnated petri plates under sterile condition. The plates were left for 30 min to allow the diffusion of extracts at room temperature. Antibiotic discs of ciprofloxacin (40 mg/ml) and ketaconazole (40 mg /ml) were used as positive control, while negative controls were prepared using the same solvent employed to dissolve the oil. Then the plates were incubated for 24 h at 37 ± 1^{o} C for antibacterial activity and 48 h at 37±1^{o} C for antifungal activity. The zone of inhibition was calculated by measuring the minimum dimension of the zone of no microbial growth around the disc.

4.10.2. Anti-microbial activity of *O.gratissimum* leaves extract:

The aqueous extract (*O.gratissimum*) of microwave oven method was tested for anti-microbial activity by dissolved in the same solvent (alcohol and water) and sterilized by filtering through 0.45 µm millipore filter. A final inoculum of 100 µl suspension containing 10^{8} CFU/ ml of each bacterium and fungus was used. Nutrient agar (antiibacterial activity) and sabouraud's dextrose agar medium (anti-fungal activity) was prepared and sterilized by an autoclave (121º C and 15 Ibs for 20 min) and transferred to previously sterilized petridishes (9 cm in diameter). After solidification, petriplates were inoculated with bacterial organisms, in sterile nutrient agar medium at 45^{o} C, fungal organisms in sterile sabouraud's dextrose agar medium at 45^{o} C in aseptic condition. Sterile Whatmann filter paper discs (previously sterilized in UV lamp) were impregnated with crude extracts at concentrations of 25, 50, 100 mg/disc were placed in the organism-impregnated petri plates under sterile condition. The plates were left for 30 min to allow the diffusion of extracts at room temperature.

Antibiotic discs of ciprofloxacin (100 µg /disc) and ketaconazole (100 µg /disc) were used as positive control, while negative controls were prepared using the same solvents employed to dissolve the plant extract. Then the plates were incubated for 24 h at 37 ± 1^{o} C for antibacterial activity and 48 h at 37±1^{o} C for antifungal activity. The zone of inhibition was calculated by measuring the minimum dimension of the zone of no microbial growth around the disc.

RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

5.1. Percentage yield of the volatile oil:

The fresh leaves were subjected to hydro distillation by using Clevenger apparatus in order to extract the volatile oil. 26 ml of volatile was obtained from about 10 kg of fresh leaves *O.gratissimum*. the weight of leaves taken, volume of oil received and percentage of the oil collected were prescribed in the Table 2.

Table 2. Percentage yield (v/w) volatile oil of *O.gratissimum* leaves

Batch No.	Wt of leaves (kg)	Volume of oil (ml)	Percentage yield (% v/w)
1.	0.100	0.2	0.2
2.	0.500	1.3	0.26
3.	1.500	4.2	0.28
4.	2.500	6.50	0.26
5.	5.400	14.2	0.262
		$\Sigma = 26.4$	Average = 0.2524

5.2. Physical properties and constants of the *O.gratissimum* volatile oil:

Organoleptic evaluation of the volatile oil was done and the following properties were noted: color, odor, taste and feel to the touch and shown in Table.3. The volatile oil for colorless to pale yellow when freshly extracted, strong aromatic odor, pungent taste and were greasy to the touch. The taste vary; sweet, mild, pungent, hot acrid, caustic or burning. They have a characteristic aroma or odor. The *O.gratissimum* volatile oil possesses the characteristics of most volatile oil.

Table 3.Organoleptic evaluation of *O.gratissimum* volatile oil

Organoleptic property	Description
Color	Colorless to pale yellow
Odor	Strong aromatic odor
Taste	Pungent
Characteristic feel	Greasy

5.3. Solubility of *O.gratissimum* Volatile oil:

The Table 4 shows the solubility of *O.gratissimum* volatile oil, was miscible in all proportions in organic solvents like ethyl alcohol, chloroform, anhydrous ether and petroleum ether. It was immiscible in water in the ratio of 0.1:0.1 but was soluble in 50.0 ml of water or no separation of phase observed. Most volatile oils are miscible in organic solvents but sufficiently soluble in water to form a saturated solution and impart its odor to the water. The *O.gratissimum* volatile oil also possesses this property of volatile oil.

Table 4. Solubility of *O.gratissimum* volatile oil

Solvent	Volume ratio (oil:solvent) ml	Description
Water	1:1	Immiscible
Water	1:5	No separation of phase but cloudy
Ethyl alcohol	1:1	Fully soluble
Petroleum ether	1:1	No separation but cloudy
Chloroform	1:1	Fully soluble
Anhydrous ether	1:1	Fully soluble

5.4. Physical constant of *O.gratissimum* volatile oil:

Physical constants serve as a means of assessing the purity and quality of the volatile oil as well as for identification. So, the specific gravity, optical activity and refractive index were determined for the isolated oil of *O.gratissimum* leaves. Table 5 shows the average values obtained from these determinations**.**

Table 5. Physical constants of the *O.gratissimum* volatile oil at 25^0C

S.No.	Specific gravity	Optical rotation	Refractive index
1	1.0005	+4.358	1.523
2	1.0007	+4.355	1.527
3	1.0007	+4.350	1.529
4	1.0005	+4.357	1.532
5	1.0007	+4.355	1.524
Average	1.0006	+4.355	1.527

The average specific gravity of the volatile oil by the specific gravity bottle method was 1.0006, the optical activity determined by Polarimeter (Model: Sipcon) was +4.355 and refractive index using a refractometer (Model: Sipcon) was 1.527. The specific gravity of official volatile oils varies approximately 0.842 to 1.172. Majority of the volatile oils are lighter than water and this indicates the *O.gratissimum* volatile oil was within the range of specific gravity of volatile oil since it was lighter than water.

The rotary power of volatile oil varies within relatively wide limits. It can either be dextrorotatory or levorotatory. This may serve as an index of purity and identity of volatile oils. The obtained specific rotation indicates that the volatile oil was dextrorotatory. The index of refraction was made by the use of in the determination of the identity and purity of volatile oil and was measured at 25^{o}C ant the index of refraction does not vary greatly with different official volatile oils. The values were between 1.4600 and 1.6100. In some case refractive index may serve in the detection of extraneous matter. The *O.gratissimum* oil was within the range of refractive index of volatile oils.

5.5. GC-MS Analysis of *O.gratissimum* volatile oil:

Chromatogram of the analysis was showed Fig.6. The total ion chromatogram (TIC) retention time about 20 min. The most composition of the oil isolate around the first 14 min of the analysis procedure and condition. The quantitative and qualitative analytical results were shown in Table.6. Totally 23 components were identified by GC-MS, representing 100% of the oil, Estragole (54.27%) was determined as the first major constituent in the oil, the second was beta-linalool (25.34%) and third (+)-camphor (9.19%). The composition with the lowest retention time is 7-methyl-3-methylene-1,6-octadiene (0.42%) at 5.535 min. and highest retention time was α-

bisabolene (2.31%) at 13.526 min. The volatile oil also contains (-)-limonene, eucalyptol, β-cis-ocimene, ɤ-terpinene, α-terpinolen, astaxanthin, β- and α-citral, copaene, β-elemene, caryophyllene, α-bergamotene, β-sesquiphellandrene, β-fernesene, α-caryophyllene, germacrene, β-selinene and α-selinene. As per the report of Lexa *et al.,* 2007, the major compounds were eugenol, cis and trans-ocimene and camphor and according to the Keita S M *et al.,* 2000, thymol was dominating, also constituted p-cymene and ɤ-terpene+t-sabiene hydrate major compounds. The Vieira R F *et al.,* 2001, was reported the chemical constituent of *O.gratissimum* volatile oil majorly of eugenol, thymol and geraniol also some flavones were xantomicrol, cirsimaritin. The study of Jirovertz L *et al.,* 2003, indicated the major compounds presented in the *O.gratissimum* oil were eugenol, β-ocimene, camphor and some countable quantity of germacrene, caryophyllene.

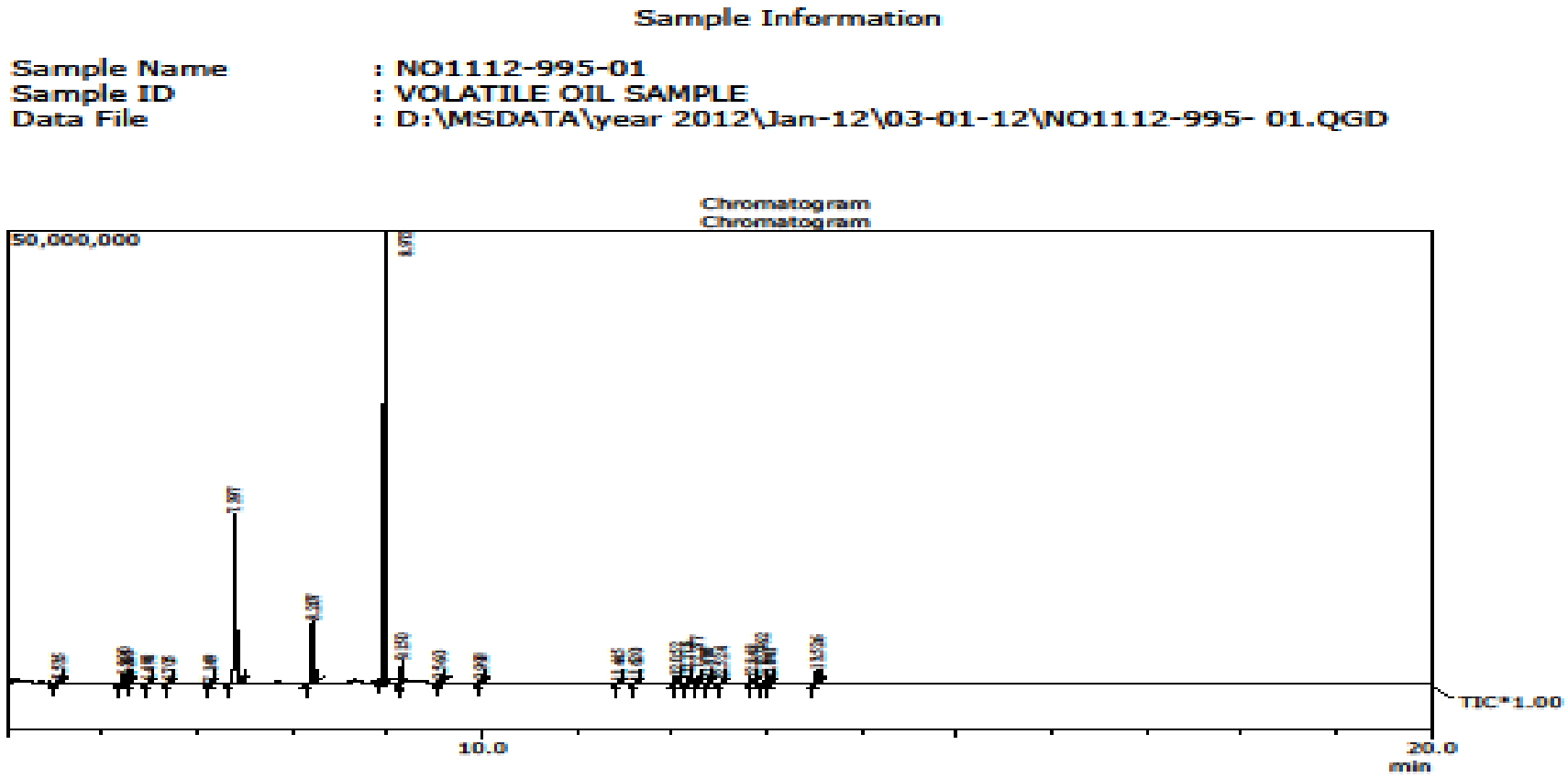
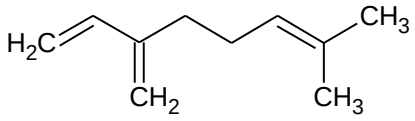


Fig. 6: GC-MS Chromatogram of *O.gratissimum* volatile oil

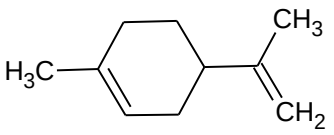
Table 6: GC-MS Report of *O.gratissimum* volatile oil

S.No.	Peak name	Formula	Mol. weight	Retention time	Peak area	% Peak area
1.	7-Methyl-3-Methylene-1,6-Octadiene	$C_{10}H_{16}$	136	5.535	437576	0.42
2.	(-)-Limonene	$C_{10}H_{16}$	136	6.220	1229429	1.17
3.	Eucalyptol	$C_{10}H_{16}$	154	6.285	483516	0.46
4.	β-cis-Ocimene	$C_{10}H_{16}$	136	6.498	148401	0.14
5.	ɤ-Terpinene	$C_{10}H_{16}$	136	6.705	205660	0.20
6.	α-Terpinolen	$C_{10}H_{16}$	136	7.149	117187	0.11
7.	β-Linalool	$C_{10}H_{18}O$	154	7.397	26708743	25.34
8.	(+)-Camphor	$C_{10}H_{18}O$	152	8.207	9690796	9.19
9.	Estragole	$C_{10}H_{12}O$	148	8.973	57212619	54.27
10.	Astaxanthin	$C_{40}H_{52}O_4$	596	9.150	1288345	1.22
11.	β-Citral	$C_{10}H_{16}O$	152	9.560	526552	0.50
12.	α-Citral	$C_{10}H_{16}O$	152	9.993	457492	0.43
13.	Copaene	$C_{15}H_{24}$	204	11.445	99365	0.09
14.	β-Elemene	$C_{15}H_{24}$	204	11.620	123036	0.12
15.	Caryophyllene	$C_{15}H_{24}$	204	12.052	1107264	1.05
16.	α-Bergamotene	$C_{15}H_{24}$	204	12.176	1084852	1.03
17.	β-Sesquiphellandrene	$C_{15}H_{24}$	204	12.277	54360	0.05
18.	β-Fernesene	$C_{15}H_{24}$	204	12.400	294625	0.28
19.	α-Caryophyllene	$C_{15}H_{24}$	204	12.524	366088	0.35
20.	Germacrene	$C_{15}H_{24}$	204	12.848	714066	0.68
21.	β-Selinene	$C_{15}H_{24}$	204	12.962	295365	0.28
22.	α-Selinene	$C_{15}H_{24}$	204	13.041	338274	0.32
23.	α-Bisabolene	$C_{15}H_{24}$	204	13.526	2433061	2.31
						100%

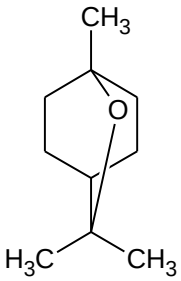
Some of chemical structures of volatile oil of *O.gratissimum* given below,



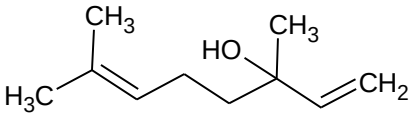
7-methyl-3-methylene-1,6-octadiene



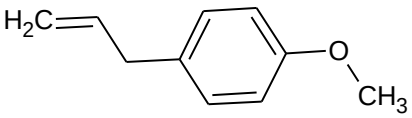
(-)-limonene



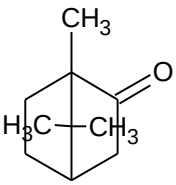
eucalyptol



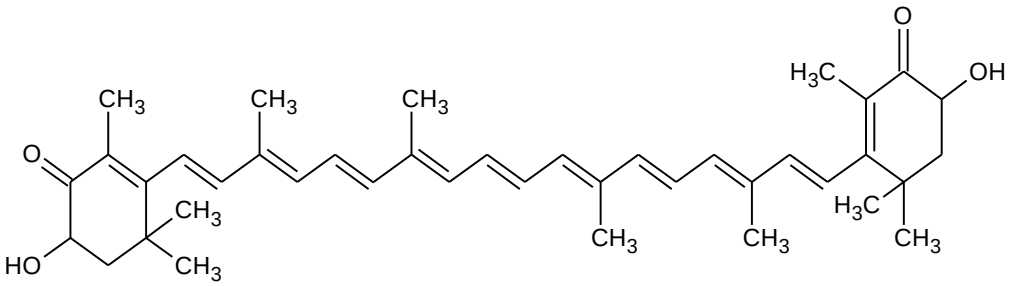
beta-linalool



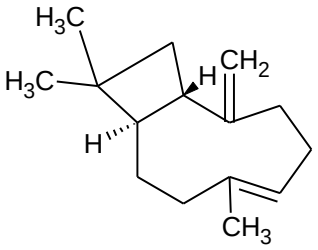
estragol



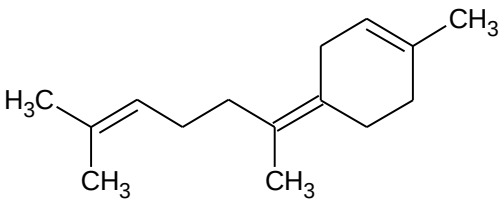
camphor



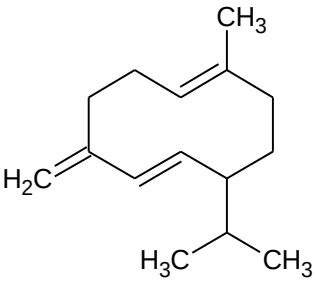
astaxanthin



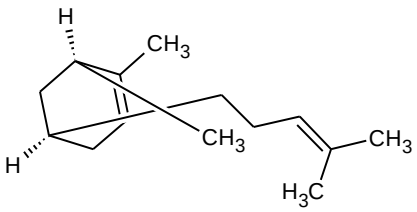
caryophyllene



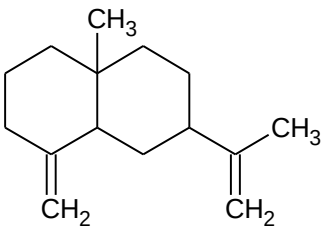
alpha-bisabolene



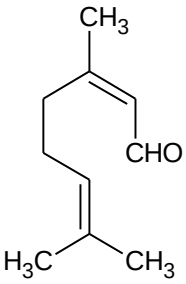
germacrene D



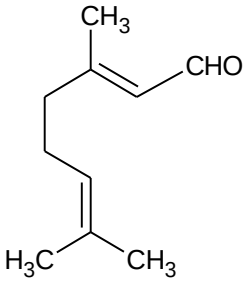
alpha-bergamotene



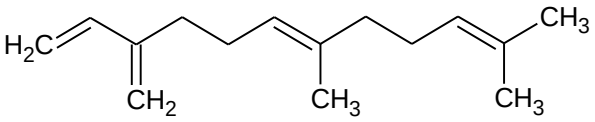
beta-selinene



trans-citral



cis-citral



beta-fernesene

Fig. 7: structure of chemical compounds present in *O.gratissimum* leaves oil

5.6. Aqueous extraction of *O.gratissimum* leaves:

The aqueous extract of *O.gratissimum* was investigated by different methods like by mechanical, by soxhlet apparatus and by microwave oven assisted hydro-distillation. Among the three methods, the mechanical extraction was revealed the presence of alkaloids, glycosides, flavonoids and phenolic compounds. At the same time soxhlet extraction gave the phyto-constituents of alkaloids, flavonoids, tannins and saponins whereas the microwave oven assisted hydro-distillation extraction showed the more compounds alkaloids, glycosides, flavonoids, reducing sugars, tannins, saponins, phenols when compared with other two methods. But no extraction showed the anthraquinones and steroids. The phytochemical constituents of the *O.gratissimum* leaves by three different methods were figured in Table 7.

Table 7. Preliminary phytochemical analysis of aqueous extract of *O.gratissimum*

S.No.	Phyto-constituents	Aqueous extract of *O.gratissimum*		
		By Mechanical method	By Soxhlet apparatus	By Microwave oven method
1.	Alkaloids	+	+	+
2.	Glycosides	+	-	+
3.	Flavanoids	+	+	+
4.	Reducing sugars	-	-	+
5.	Tannins	-	+	+
6.	Anthraquinones	-	-	-
7.	Saponins	-	+	+
8.	Phenols	+	-	+
9.	Steroids	-	-	-

+ = Present, - = Absent.

5.7. Quantitative estimation of aqueous extraction of *O.gratissimum* leaves:

The chemical composition of aqueous extract of *O.gratissimum* by microwave oven extraction was quantified for their phytochemicals by different procedures and they were showed in Table 8. Saponin was constituted as more account in the extract whereas the phenolic compound was the low quantity.

Table 8. Quantitative estimation of *O.gratissimum* aqueous extract:

S.No.	Composition	Values in mg/g
1.	Total phenols	1.724
2.	Flavonoids	0.984
3.	Saponins	1.764
4.	Alkaloids	1.675
5.	Reducing sugars	1.563
6.	Non-reducing sugars	0.967
7.	Tannins	0.934

5.8. Physico-chemical constants of *O.gratissimum* leaves.

The aqueous extract of *O.gratissimum* leaves by microwave oven extraction was investigated for their physico-chemical constants. The total ash was constituted high account such as ash value 1.57%, very less amount was sulphated ash 0.76%, acid insoluble ash 0.89 and water soluble ash 0.98%, which have shown in Table 9.

Table 9. Proximate composition of *O.gratissimum* leaves:

S.No.	Composition	Values (%)
1.	Total ash value	1.57
2.	Sulphated ash	0.76
3.	Acid insoluble ash	0.89
4.	Water soluble ash	0.98

5.9. Qualitative analysis of elements in *O.gratissimum* leaves:

The leaves of *O.gratissimum* were investigated for their element analysis and they have shown in Table 10. Few of elements were presented in the analyzed leaves like calcium, iron, sulphate, phosphate, magnesium, sodium and potassium at the same time carbonate and nitrate showing the absence.

Table 10. Elemental composition of *O.gratissimum* leaves:

S.No.	Elements	Specification
1.	Calcium	+
2.	Iron	+
3.	Sulphate	+
4.	Phosphate	+
5.	Magnesium	+
6.	Sodium	+
7.	Potassium	+
8.	Carbonate	-
9.	Nitrate	-

+ = Present, - = Absent.

5.10. Quantitative estimation of *O.gratissimum* leaves:

The leaves of *O.gratissimum* were quantified and they have shown in Table 11. The leaves contain calcium, magnesium, sodium and potassium with accountable amounts in the leaves. The calcium was constituted high content as 2.34%, as well as sodium was 0.92% as lower content and magnesium 1.56%, potassium 1.78% were contained specified amount in the leaves. Finally it revealed that the leaves contained iron and phosphate also with respective countable account.

Table 11. Quantitative estimation of elemental composition of *O.gratissimum* leaves

S.No.	Elements	Value (%)
1.	Calcium	2.34
2.	Magnesium	1.56
3.	Sodium	0.92
4.	Potassium	1.78

5.11. Identification of isolated compound:

The isolated compound from the column chromatography was quercetin and which was identified by following spectral methods.

5.11.1. IR-Spectrum of Quercetin:

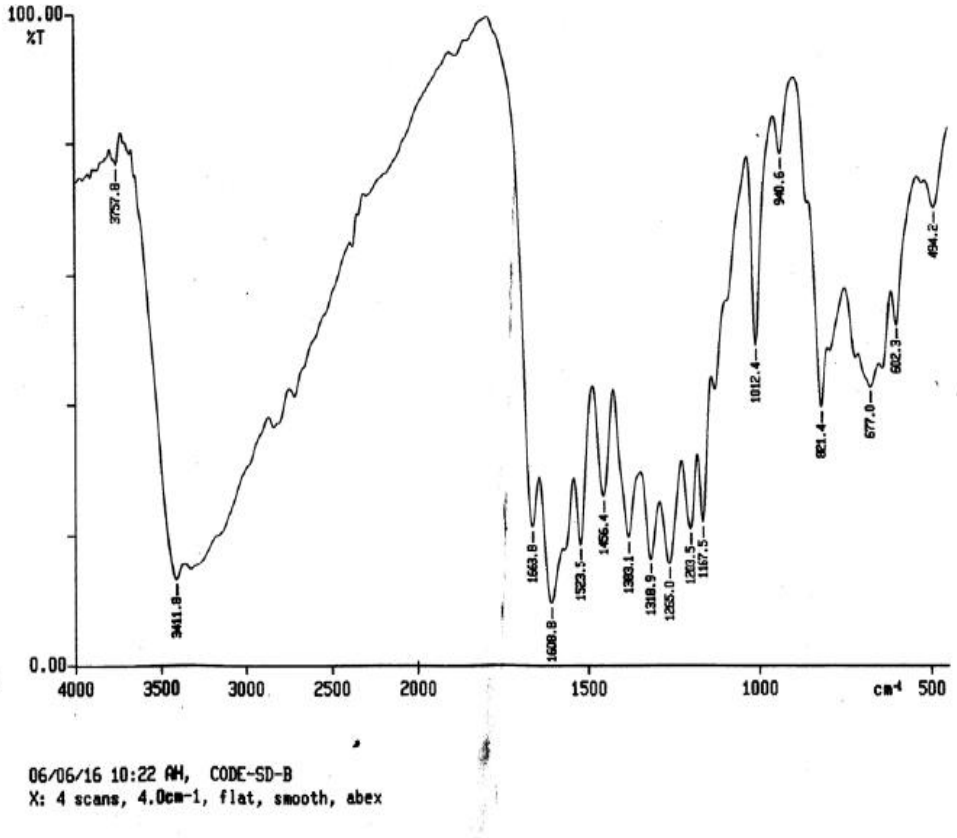


Fig. 8: IR-Spectrum of Quercetin

Table 12. IR Values of the Quercetin:

1	3411 cm^{-1}	O-H stretching vibration of Phenols
2	1663.1 cm^{-1}	C=O Aryl Ketonic stretch
3	1608.8 cm^{-1}, 1523.5cm^{-1}, 1496 cm^{-1}	C-C Aromatic ring stretch
4	1383.1 cm^{-1}	In plane O-H bending of Phenols
5	1318.9 cm^{-1}	In plane bending of C-H bond in Aromatic Hydrocarbon
6	1265 cm^{-1}	C-O stretch of Aryl ether
7	1203 cm^{-1}	C-O stretch of Phenol
8	1167 cm^{-1}	C-CO-C stretch and bending in Ketone
9	940.6, 821.4, 677, 602.3 cm^{-1}	Out of plane C-H bending of Aromatic Hydrocarbon

5.11.2. MS-Analysis of the Quercetin:

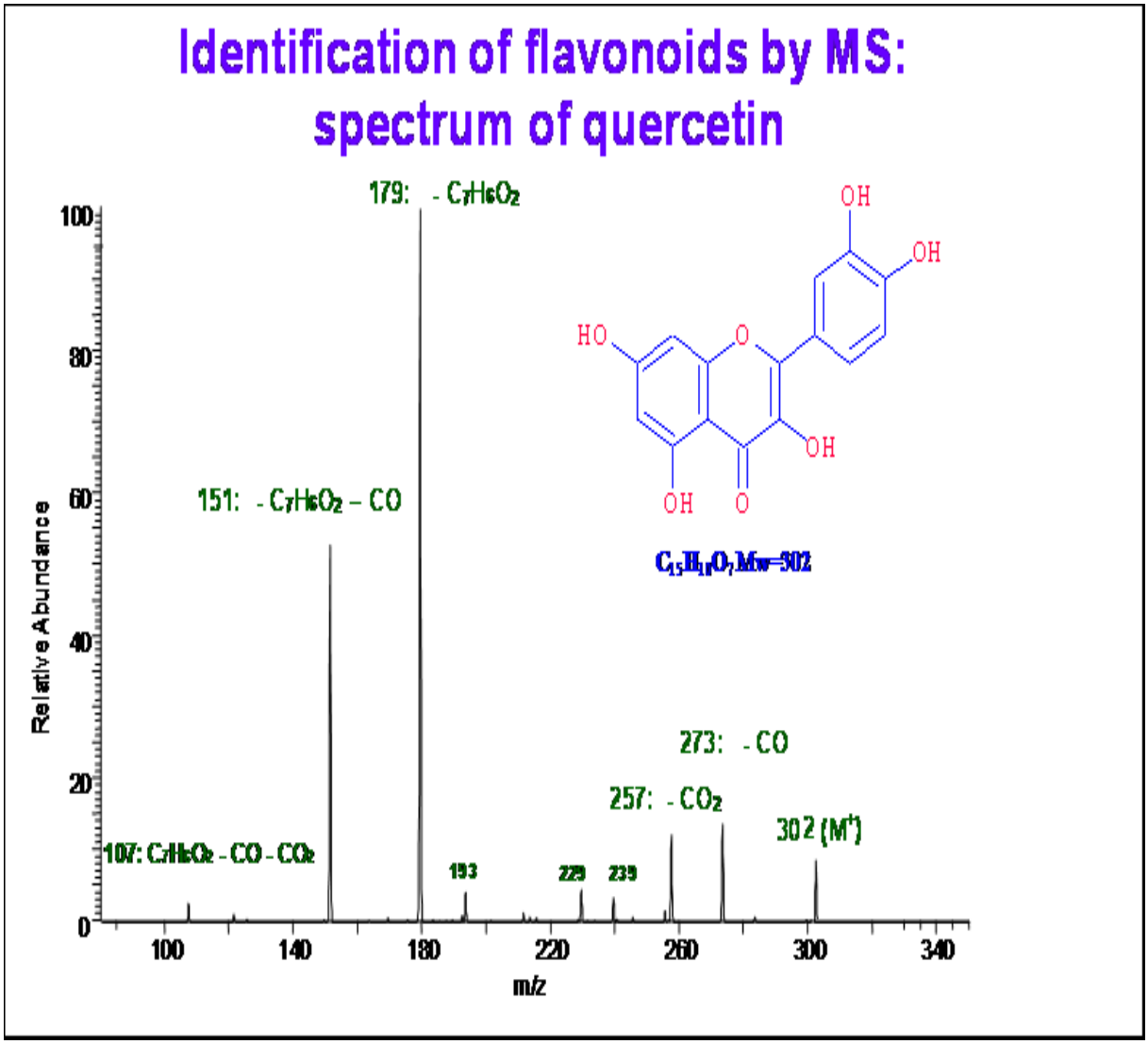


Fig. 9: MASS spectrum of Quercetin

5.11.3. NMR-Analysis of Quercetin:

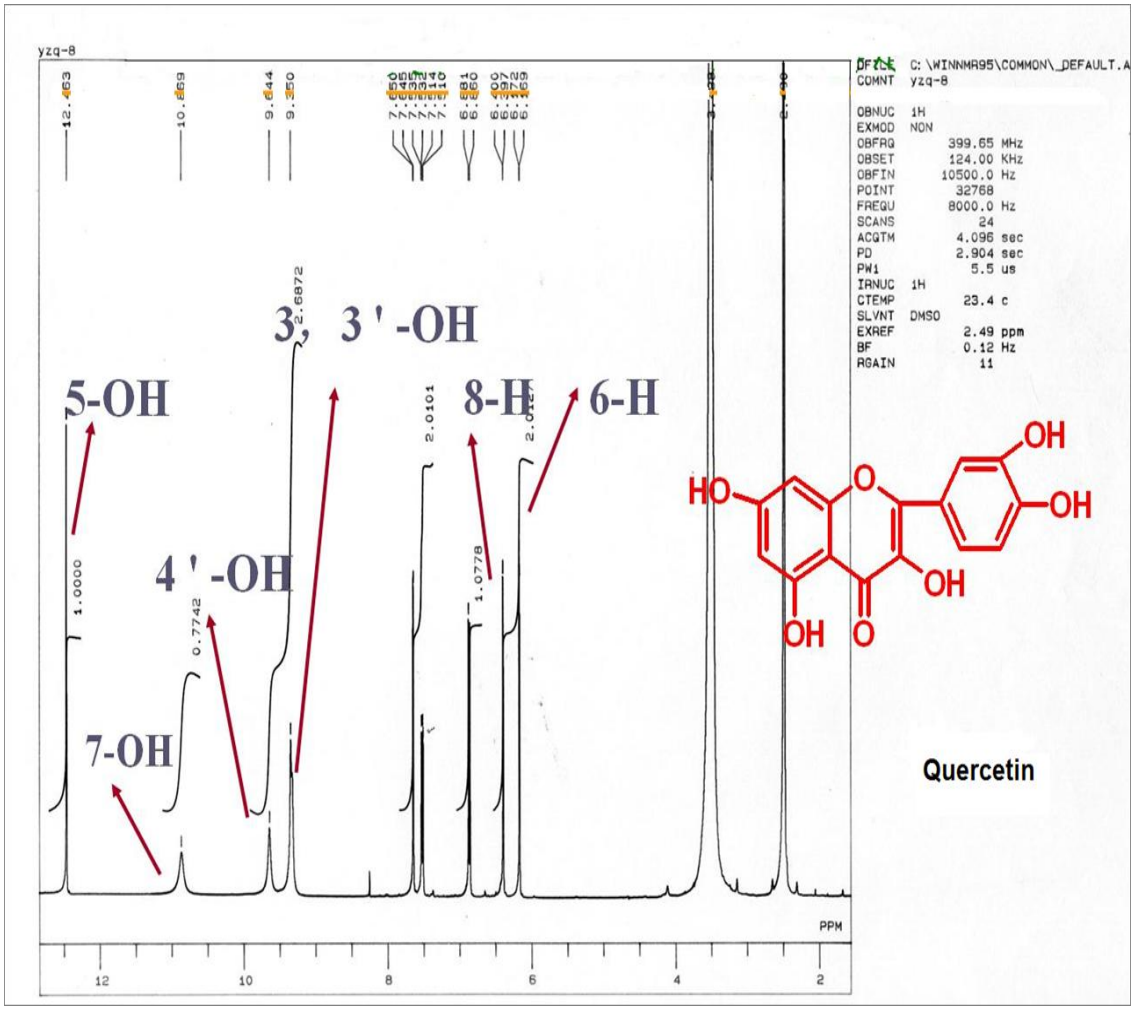


Fig. 10: NMR- Spectrum of Quercetin

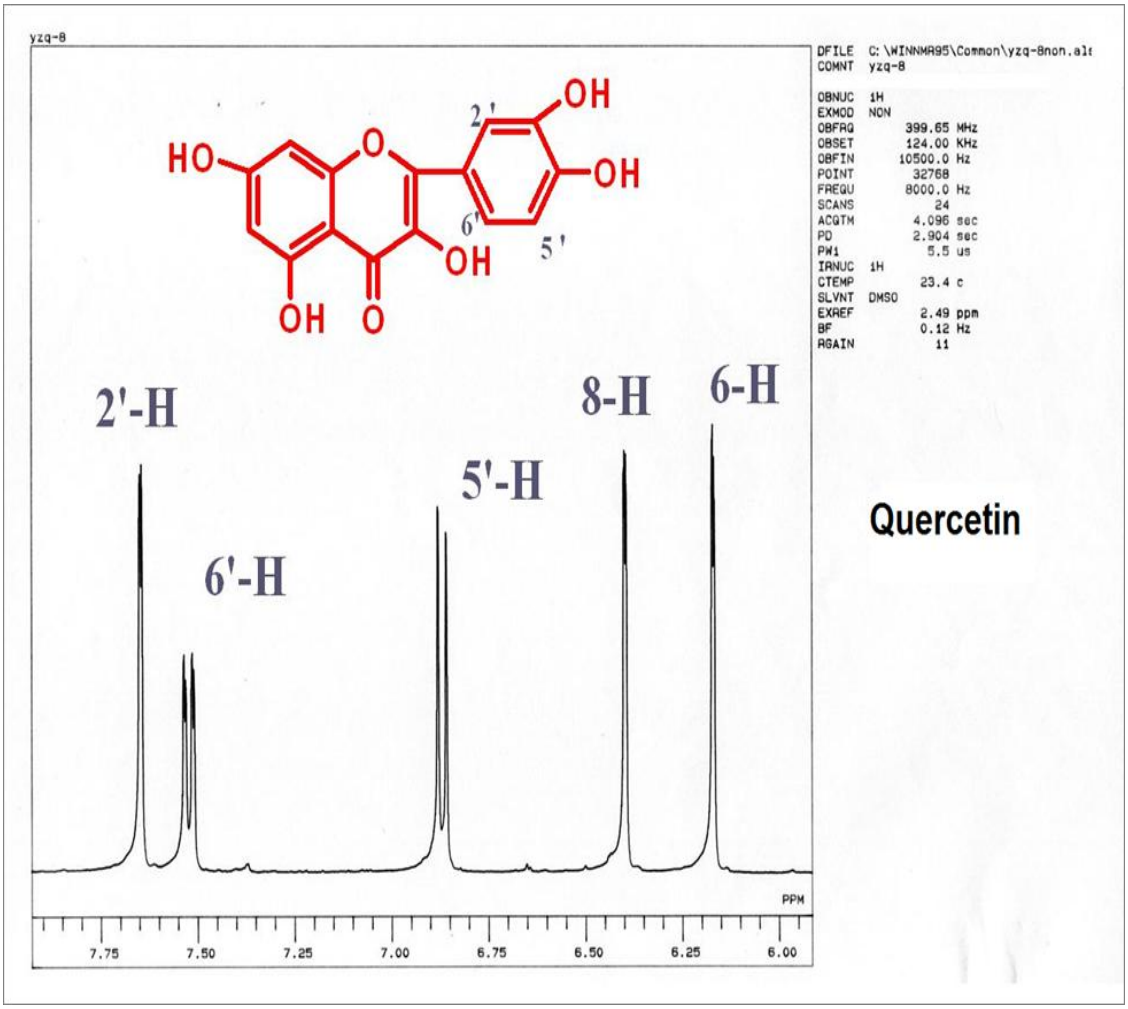


Fig. 11: NMR- Spectrum of Quercetin (Contd.)

Table 13. NMR Values of Quercetin

Protons signal	**No.of protons**	**Assignment**	
6.2	1	Doublet	H-6
6.5	1	Doublet	H-8
6.9	1	Doublet	H-5’
7.7	1	Doublet	H-6’
7.9	1	Doublet	H-2’

5.12.1. Anti-microbial activity of volatile oil of *O.gratissimum* leaves:

The Disc diffusion method was used to determine the anti-microbial activity of the volatile oil of *O.gratissimum* leaves and shown in Table.14. The anti-microbial activity of oil was screened against some bacterial strains (*P.aeruginosa, E.coli, P.vulgaris, B.subtilus and S.aereus*) and against a fungus organisms *C.albicans* (Fig.12 and 13) at different concentrations 25, 50 and 100 µl per disc and compared with positive controls ciprofloxacin for antibacterial activity whereas ketaconazole for antifungal activity respectively. The extract exhibited marked anti-microbial activity against the tested microorganisms. The oil exhibited marked anti-microbial activity against the tested microorganisms.

Table 14. Anti-microbial activity of *O.gratissimum* leaves volatile oil

S.No.	**Micro-organisms**	**Zone of inhibition**				
		25 µl	**50 µl**	**100 µl**	**Ket.**	**Cip.**
1.	*P.aeruginosa*	15	16.5	18	---	20
2.	*Candida albicans*	17.3	19	20.2	21	---
3.	*E.Coli*	14	15.5	17	---	19.3
4.	*P.vulgaris*	16.2	17.5	19.3	---	21.5
5.	*Bacillus subtilus*	18.4	20	22	---	23
6.	*Staphylococcus aereus*	17	18.5	20.5	---	21

Ket. = Ketakonazole and Cip. = Ciproflaxacin as standards

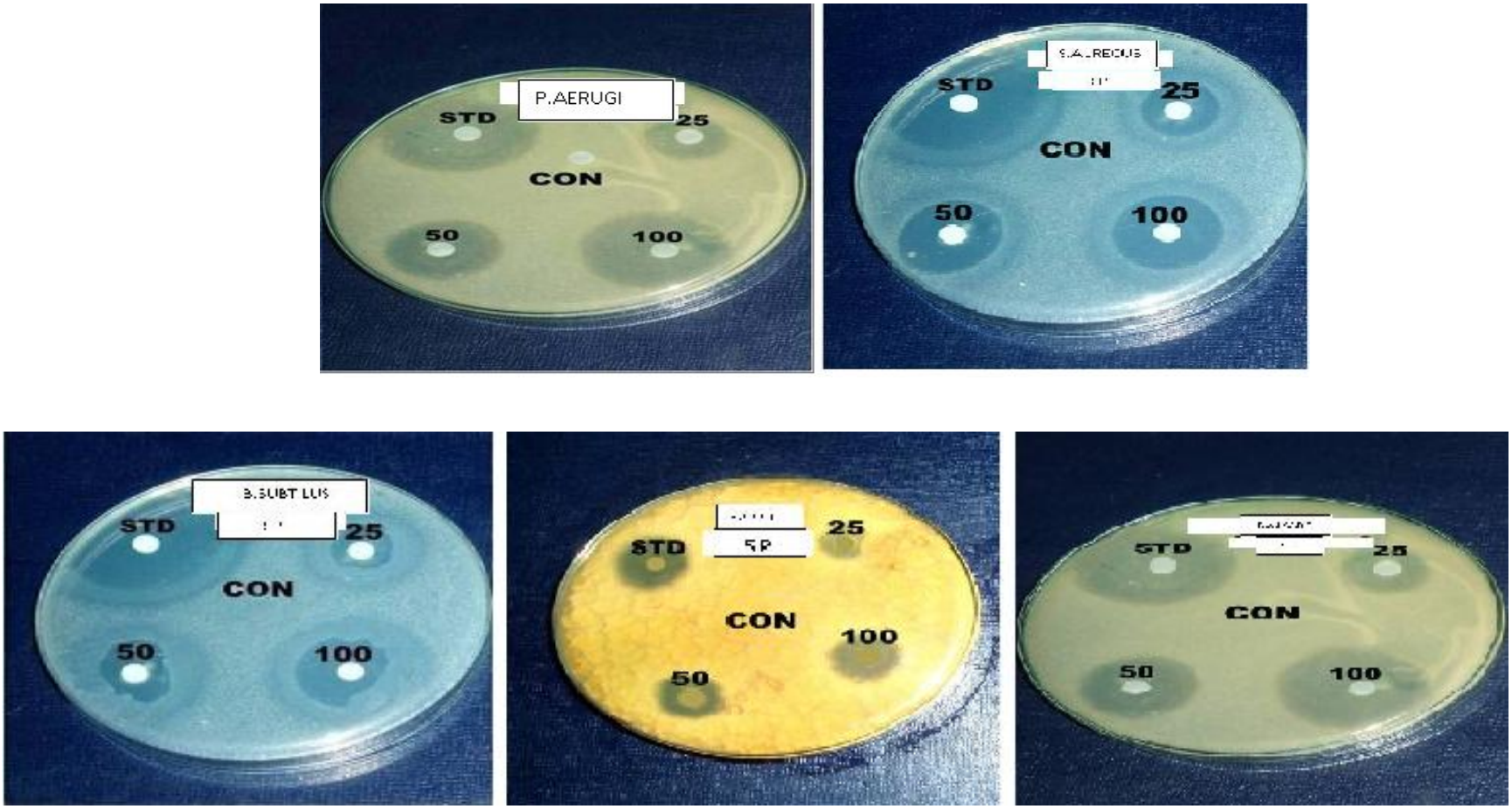


Fig. 12: Anti-bacterial activity of *O.gratissimum* oil

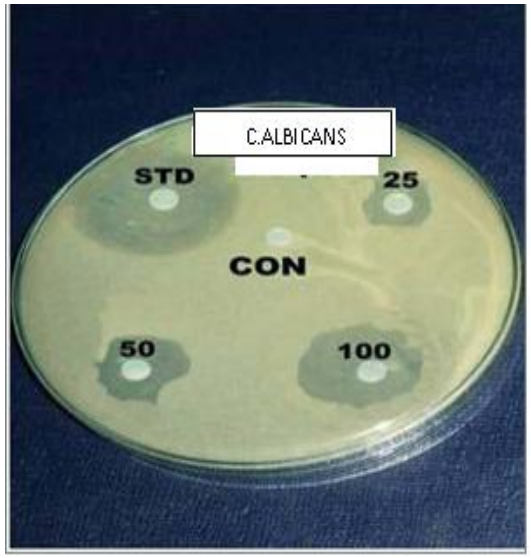


Fig. 13: Anti-fungal activity of *O.gratissimum* oil

5.12.2. Anti-microbial activity of aqueous extract of *O.gratissimum* leaves:

The Disc diffusion method was used to determine the anti-microbial activity of the aqueous extract of *O.gratissimum* leaves by microwave oven method and shown in Table.15. The anti-microbial activity of the extract was tested against some bacterial (*Basillus cereus, Basillus subtilus, Staphylococcus)* and fungal (*Aspergillus flavus, Candida albicans, Aspergillus niger*) micro-organisms (Fig.14 and 15) at different dilution of the extract as 25, 50 and 100 µg and compared with positive controls, ciprofloxacin for antibacterial activity whereas ketaconazole for antifungal activity respectively. The extract exhibited marked anti-microbial activity against the tested microorganisms.

Table 15. Screening of the anti-microbial activity of *O.gratissimum* aqueous extract

S.No.	Micro-organisms	Zone of inhibition				
		25 µg	**50 µg**	**100 µg**	**Ket.**	**Cip.**
1.	*Aspergillus flavus*	12	15	17	19	---
2.	*Candida albicans*	12	14	16	20	---
3.	*Aspergillus niger*	10	12	15	21	---
4.	*Basillus cereus*	11	13	14	---	20
5.	*Basillus subtilus*	13	15	17	---	21
6.	*Staphylococcus pyogens*	13	16	18	---	22

Ket. = Ketakonazole and Cip. = Ciproflaxacin as standards.

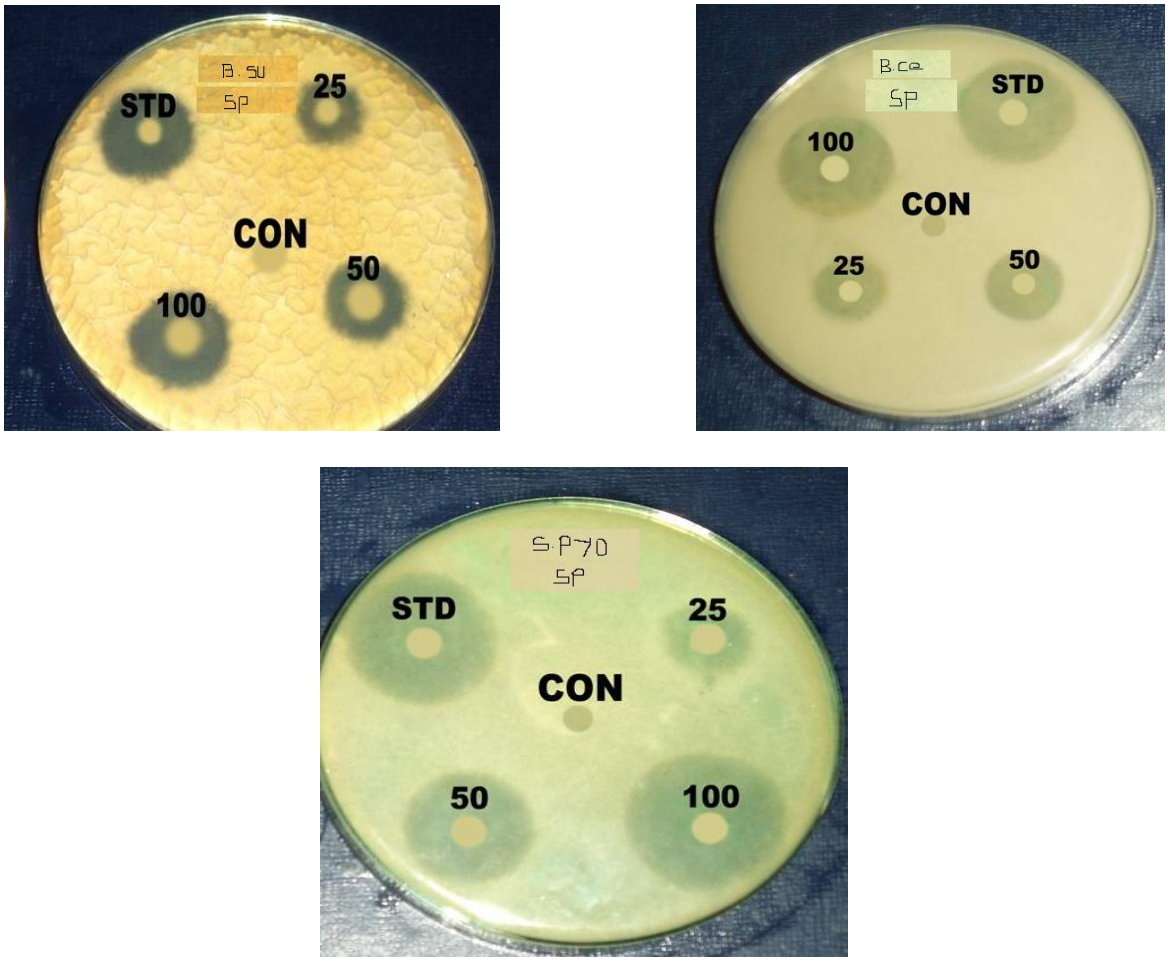


Fig. 14: Anti-bacterial activity of aqueous extract of *O.gratissimum*

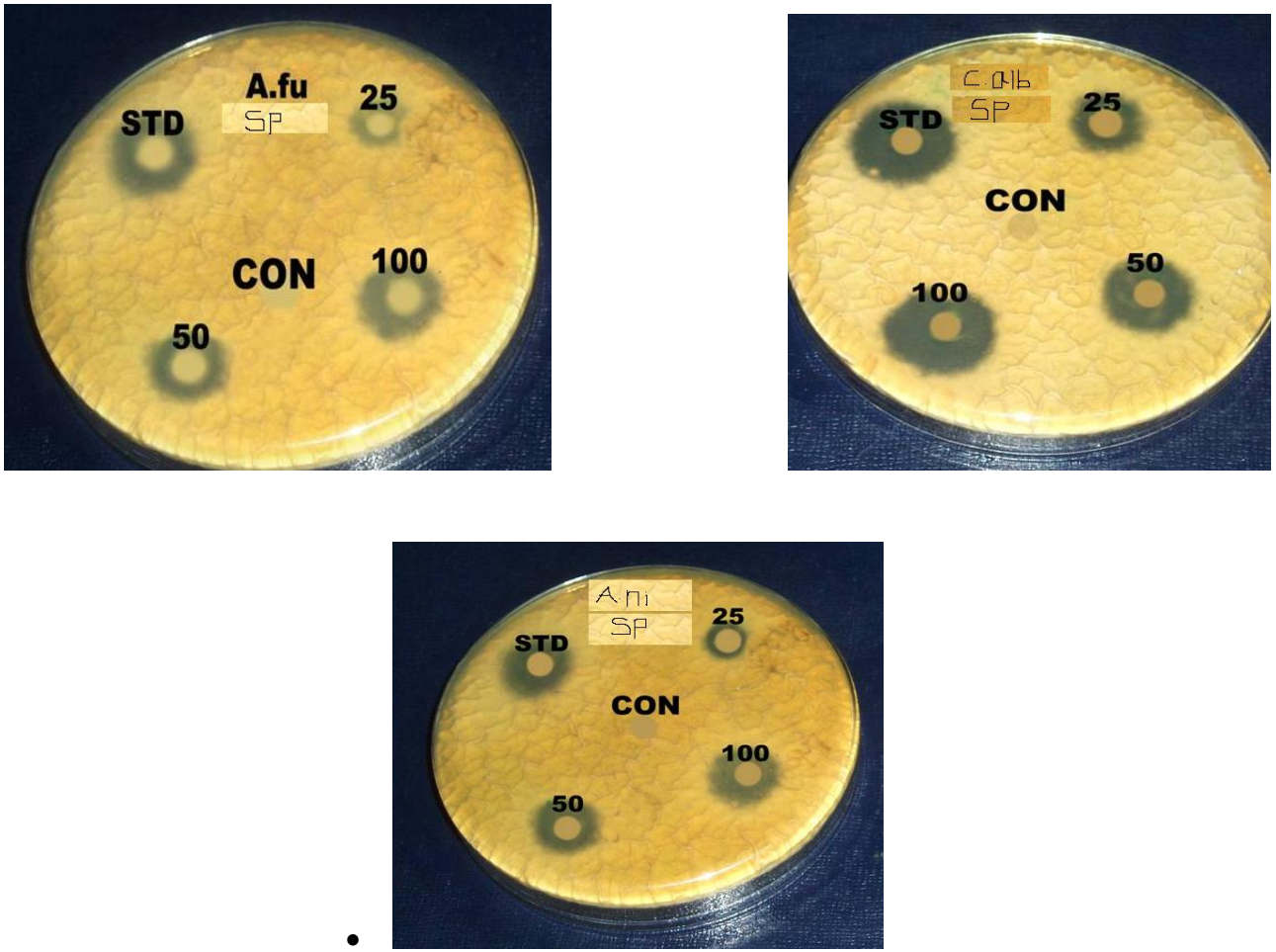


Fig. 15: Anti-fungal activity of aqueous extract of *O.gratissimum*

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

Medicinal plants have played a vital role in treating diseases and in promoting health of mankind for a long time. They continue to become an important source of medicinal agents. The volatile oil of plant leaves was taken by Clevenger apparatus and the physical constants such as specific gravity, refractive index, optical rotation obtained confirms that the *O.gratissimum* oil is pure and posing the characteristics of a volatile oil. The total ion chromatogram retention time is about 20 min. Estragole, linalool and camphor was the major compounds.

On the extraction of *O.gratissimum* leaves by different methods (mechanical, by soxhlet apparatus and by using microwave oven), the microwave oven method offered more compounds when compared with other methods.

The leaves of studied plant contained accountable amount of phyto-chemicals and mineral contents.

The anti-microbial activity of the *O.gratissimum* leaves oil and its aqueous extract having significant activity against tested bacterial and fungal strains when compared with standards ciprofloxacin (anti-bacterial) and ketaconazole (anti-fungal). The isolated compound Quercetin from column chromatography was identified by IR, NMR and Mass spectroscopy analysis and which may be responsible for the anti-microbial activity.

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APPENDIX

8.1. Certificate of authenticated plant *O.gratissimum*

भारत सरकार / GOVERNMENT OF INDIA
पर्यावरण एवं वन मंत्रालय / MINISTRY OF ENVIRONMENT & FORESTS
भारतीय वनस्पति सर्वेक्षण/ BOTANICAL SURVEY OF INDIA
दक्षिणी क्षेत्रीय केन्द्र / Southern Regional Centre
टी.एन.ए.यू. कैम्पस / T.N.A.U. Campus
लाउली रोड / Lawley Road
कोयंबत्तूर/ Coimbatore - 641 003
टेलीफोन / Phone: 0422-2432788, 2432123, 2432487
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ई-मैल /E-mail id: sc@bsi.gov.in
bsisc@rediffmail.com

सं. भा.व.स./द.क्षे.के./No. BSI/SRC/5/23/2011-12/Tech. - 576 दिनांक/Date: 13 July 2011

सेवा में / To

Mr. A. Pandiyan
IInd M. Pharm.
Department of Pharmaceutical Chemistry
Adhiparasakthi College of Pharmacy
Melmaruvathur
Kancheepuram Dist.
Tamil Nadu

महोदय/Sir,

The plant specimen brought by you for identification is identified as *Ocimum gratissimum* L. - LAMIACEAE

धन्यवाद/Thanking you,

भवदीय/Yours faithfully,

13/7/11

(डॉ. जी.वी.एस. मूर्ति /Dr. G.V.S. Murthy)
वैज्ञानिक 'एफ' एवं कार्यालय अध्यक्ष /
Scientist 'F' & Head of Office

वैज्ञानिक 'एफ' एवं कार्यालय अध्यक्ष
Scientist 'F' & Head of Office
भारतीय वनस्पति सर्वेक्षण
Botanical Survey of India
दक्षिणी क्षेत्रीय केन्द्र
Southern Regional Centre
कोयंबत्तूर / Coimbatore - 641 003